CHAPTER - 2

Chemical Investigation of
Bacopa monnieri 'Linn'
INTRODUCTION

_Bacopa monnieri_ (Linn) N.O. Scruphluriaceae local name 'Brahmi' is a very important drug of Ayurvedic. It is widely distributed throughout India in wet places and Ceylon. _Bacopa monnieri_ is a large creeping plant with a small particularly in flower, the leaves are ovate trilobed and small.

It is used in the indigenous system of medicine in the treatment of mental ailment and epilepsy and a potent nerve tonic, cardiotonic and diuretic. It also improve memory and removes hoarseness. Importance of this plant in the indigenous system of medicine led us to carry out a systematic chemical investigation of plant.

Shastray and Dalla\(^1\) isolated a variety of compounds including aspartic acid, glutamic acid and \(\alpha\)-alanine. Sample of drug from different sources were analysed by Basu\(^2\) (1931). It was found that all the specimen contained an alkaloid in varying proportion. The alkaloid could be extracted by macerating the drug with ether-chloroform mixture in cold. Only about .01% of the alkaloid could be isolated. Basu\(^3\) from the whole plant isolated three new bases, \(B_1\) oxalate mp 330°C, platinic chloride mp 100-101°C, \(B_2\) oxalate mp 180°C platinic chloride mp above 300°C, hydrochloride mp 26°C, \(B_3\) oxalate mp 204°C. The alkaloid was named as herpestrin,
a dibasic acid mp 116-17°C, a trace of oil matter soluble in alcohol, two resins, an organic acid were also isolated. A triterpene saponin named monnoirin mp 263-5°C was also isolated from the plant. The total ash 14.58% and the acid insoluble ash (1.00%) showed the presence of Cl⁻, SO₄²⁻, Na⁺, K⁺, Fe³⁺ and Ca²⁺ in addition to silica

ISOLATION AND CHARACTERISATION OF CONSTITUENTS

The extensive use of Bacopa monnieri (Linn) in the indigenous system of Indian medicine and the observed astringent activity reported in its acetone extract directed us to undergo a detailed chemical investigation of the plant in the expectation of isolating the active principle responsible for its medicinal importance.

The detailed studies have resulted in the isolation of following constituents as given in table -

<table>
<thead>
<tr>
<th>Constituents of Bacopa monnieri</th>
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<tbody>
<tr>
<td>Constituents</td>
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<td>-----------------------------</td>
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<tr>
<td>1. Compound A</td>
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<tr>
<td>(Bacoside-A)</td>
</tr>
<tr>
<td>a) Bacogenin A₁</td>
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<tr>
<td>b) Bacogenin A₂</td>
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<tr>
<td>c) Bacogenin A₃</td>
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<td>d) Bacogenin A₄</td>
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</table>
2. **Compound B**  
(Bacoside-B)  
**203** \( \text{C}_{47}\text{H}_{76}\text{O}_{19} \)

3. **Compound C**  
(D-mannitol)  
**166** \( \text{C}_{6}\text{H}_{11}\text{O}_{6} \)

4. **Compound D**  
(Betulinic acid)  
**315** \( \text{C}_{30}\text{H}_{48}\text{O}_{3} \)

5. **Compound E**  
(Stigmasterol)  
**170** \( \text{C}_{29}\text{H}_{48} \)

6. **Compound F**  
(β-Sitosterol)  
**137** \( \text{C}_{29}\text{H}_{50} \)

7. **Compound G**  
(Gallotannin)  
**233-34** \( \text{C}_{48}\text{H}_{36}\text{O}_{33} \text{H}_{2} \text{O} \)

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1) **ISOLATION OF COMPOUNDS**

The whole plant material freshly collected and air dried was moistened with water and extracted with alcohol. The alcohol extract was concentrated under reduce pressure and left in cold when a crystalline powder was deposited, recrystallised from dilute methanol as colour less needles as compound C.

The filtrate was diluted to 60% ethanol (fraction x) and repeatedly partitioned with benzene (fraction y). The ethanol fraction 'x' was treated with excess of lead acetate in order to remove tannin. The lead salt was filtered and the residue lead was removed from the filtrate with \( \text{H}_2\text{S} \). The whole solution was concentrated and a vicious slimmy material precipitated which dissolved in alcohol, ether and petroleum.
ether. All these solutions were concentrated in vacuo and residue was macerated with acetone and filtered. The acetone insoluble powder was partitioned between butanol and water. During concentration of butanol solution in vacuo, a precipitate settled down (F-I). The filtrate was again concentrated and precipitated with acetone and ether which yield a powder (F-II). The mother liquor was concentrated to dryness when a brown powder (F-III) was obtained. These fractions obtained above were chromatographed on paper chromatogram in different solvent and identified as compound A.

The acetone soluble fraction from above left in cold for several days when a solid settled down which was filtered and washed with acetone (F-IV). The filtrate was concentrated almost to dryness and the hygroscopic resinous material was macerated with ethyl acetate. The insoluble material was dissolved in alcohol fractionally precipitated with ethyl acetate and ether. The precipitate thus obtained was warmed with alcohol cooled and filtered when a brown powder (F-V) was obtained. The alcoholic filtrate and freed of solvent to a brownish water soluble semi-solid (F-VI). These portions were chromatographed on paper chromatogram in different solvent and identified as compound B.

Compound A and B on acid hydrolysis with 8% sulphuric acid in methanol-water 60:40 solution yielded two fractions, a glycone fraction which contain sugar i.e. glucose
and arabinose while other aglyconic fraction contain a mixture of sapogenins as bacogenin \( A_1, A_2, A_3 \) and \( A_4 \).

The total benzene fraction \( Y \) obtained in the fractionation of original plant extract was concentrated, saponified and extracted with ethyl acetate. This solution on concentration formed a thick gel which was cooled and filtered. The filtrate gave a further quantity of the gelatinous precipitate on concentration. The total precipitate was again dissolved in hot ethyl acetate, cooled and filtered. A crystalline powder thus obtained as compound \( D \).

The rest mother liquor obtained above was concentrated to dryness and residue was chromatographed on alumina. The elutions from 11–19 fractions were mixed, crystallised from methanol and rechromatographed over alumina using benzene: chloroform (1:2) as eluants. Colourless needles were obtained yielding a mixed crystals compound \( E \) and \( F \) and were resolved by suitable procedure.

The solid material from fraction 26–27 was mixed with little warm acetone, cooled and filtered. The powder crystallized from alcohol as colourless needles. It was identified as compound \( D \).

The residue left after extraction of material with chloroform was extracted with acetone. Acetone extract was concentrated and ether was added, some impurities which settled down first were removed and to the decanted solution
more ether was added when brown precipitate was obtained. This on repeated crystallisation from acetone and ether mixture gave an almost creemish, microcrystalline, chromatographically, homogeneous which on TLC gave \( R_f 0.32 \) (Solvent Benzene:Methanol 1:1) sprayed with \( I_2 \) identified as compound G.

(2) **CHARACTERISATION OF COMPOUND A**

Bacoside A is a colourless crystalline powder mp. 250-1°C (decomp.). It is soluble in alcohol and insoluble in ether, acetone etc. It forms a vigorous forth in aqueous suspension \([\alpha]_D -42^\circ\) (80% ethanol). Bacoside A, on acid hydrolysis for 4 hours gave a mixture of aglycones and three sugars. \( R_f 0.15, 0.20 \) and 0.24 on paper chromatography. The three sugars components were separated by ion exchange chromatography over Dowex - 50W (Li\(^+\) form). The sugars corresponding to \( R_f 0.20 \) and 0.24 were identified as D-glucopyranose and L-arabinopyranose by optical rotation, Osazone formation and by paper chromatography, alongside authentic sample in different solvent system. The third component \( R_f (0.15) \) was found to be a disaccharide since on hydrolysis it gave glucose and arabinose. When the hydrolysis of bacoside A was prolonged to 6 hours hydrolysis it was revealed that bacoside A consist of aglycone 62% and sugar 35.3%. Bacoside A is a laevorotatory, it does not undergo
alkaline hydrolysis which suggests that the saponin is a glucoside and not an ester. Emulsin and pilzferment C_{1290} do not hydrolyse bacoside A. This indicating that in the disaccharide moiety arabinose is the terminal sugar. Since the prefered position for the attachment of the carbohydrate moiety in triterpenoid glucoside is C_3 by analogy the position of sugar linkage in bacoside A can be assumed to be the same. This is substantiated by the fact that the only other available position, the hydromethyl group at C_{10} of the aglycone is hindered and would therefore not be expected to carry the disaccharide residue the tentative formation can be shown as - I

\[
\text{Arabinose glucose-} \quad \text{(I)}
\]

Acid hydrolysis of bacoside A gave a complex mixture of aglycones which were separated into ether soluble and ether insoluble fractions. TLC showed five major spot in the ether soluble fraction and a long streak in the insoluble fraction. So far no homologous product has been obtained from the later portion by the different purification technique employed by us, various method were tried from the isolation of different component of the ether soluble fraction e.g.
fractional precipitation from solvent mixture and chromatography on various adsorbants. The attempted separation into ketonic and non-ketonic fractions by Girard reagent was only partially successful. The separation of the major component was finally achieved by chromatography over alumina and then rechromatography of the separated over silica gel using frontal elution technique. This lead to the isolation of four crystalline aglycone which have been designated as bacogenin A$_1$ mp. 242°C, bacogenin A$_2$ mp. 222°C, bacogenin A$_3$ mp. 190°C and bacogenin A$_4$ mp. 170°C.

The elution pattern of the bacogenin along with other data indicated that A$_1$ would be the most polar of the four aglycone and may thus be the true aglycone of bacoside A. The following studies were, therefore carried out to determined its constitution.

**Bacogenin A$_1$**

Bacogenin A$_1$ C$_{30}$H$_{50}$O$_4$ gave a positive Nollers reaction which along with the IR absorption in the methyl and methylene bending region showed that it belonged to the triterpenoids class of compounds. A yellow colour with tetra nitromethane indicated unsaturation in the molecule and a negative hydroxamic acid test showed the absence of ester or lactone grouping. The IR absorption bands at 3450, 1725 and 828 cm$^{-1}$ corresponded to hydroxyl group, a five membered ring
FIG. 1 IR SPECTRA OF BACOGENIN A₁
ketone and a trisubstituted double bond respectively. It did not show any absorption in UV. Bacogenin A₁ formed a diacetate and since the acetate still gave an IR absorption at 3440 cm⁻¹. The presence of a non acylable hydroxyl group was indicated. It was not oxidised with periodate which showed that the hydroxyl groups were not situated at glycolic position in the molecule.

On reduction with lithium aluminium hydride, bacogenin A₁ gave a product mp. 252° C which showed all the IR bands of the parent compounds except that at 1725 cm⁻¹, indicating the complete reduction of the carbonyl group. The resultant secondary hydroxyl group was resistant to acetylation as shown by the fact that the reduced product formed only a diacetate instead of the expected triacetate and the diacetate still showed strong absorption at 3450 cm⁻¹ due to hydroxyl group. This would indicate the axial character of this hydroxyl group which would be obtained by the reduction of a hindered keto group. The presence of such a keto group was indicated by the incomplete reaction of bacogenin A₁ with Girard reagent and the formation of as 2,4-dinitrophenyl-hydrazone and an oxime in the low yield.

Thus the nature of the four oxygen functions in the molecule may be summarised as three hydroxyl groups - two are acetylatable while the third is resistant to acetylation and a keto group in a cyclopentane ring.
Bacogenin A₁ as also its lithium aluminium hydride reduction product consumed one mole of hydrogen each on catalytic hydrogenation thereby confirm the presence of one double bond which is considered to be trisubstituted on the basis of the IR spectrum. It is therefore concluded that bacogenin A₁ belonging to class of tetracyclic triterpenoid. The NMR spectra of bacogenin A₁ and its diacetate were examined in detailed and have revealed the basic skeleton and position of the substituents. Assuming that the region between 200 and 350 cps contained only four protons, the integration of the spectrum of acetyl bacogenin A₁ gave a total number of 54 protons which confirms our molecular formula obtained on the basis of analytical data. The spectra resemble those of tetracyclic triterpenes belonging to the dammarane series.

In NMR spectra of both bacogenin A₁ and its diacetate there is an olefinic proton giving a split signal at 320 cps which is coupled to the methyl group at 96 and 91 cps upon spin decoupling it collapse to a sharp singlet thus confirming the partial structure II.

The broad signal at 192 cps in the spectrum of bacogenin A₁ indicates the presence of a single proton on a carbon atom bearing an oxygen function that such is the case is confirmed by the corresponding signal of acetyl bacogenin A₁ where it shifts by 60 cps to the lower field (region of
FIG. 2 NMR SPECTRA OF BACOGENIN A1

OH, $\text{F}_3\text{C} \cdot \text{COOH}$ added

- 242
- 235
- 83
- 91
- 52
- 46.5

Cps
255 cps), thus suggesting the presence of a hydroxyl group at position 3 of the ring as in a normal triterpene III.

\[ \text{(II)} \]
\[ \text{(III)} \]
\[ \text{(IV)} \]

The quartet with signal at 223, 235, 242, 254 cps in bacogenin A$_1$ shifts to 243, 255, 268, 280 in the acetyl derivative. This expected shift of almost 20 cps downfield, upon acetylation is indicative of an oxymethylene group on a fully substituted carbon atom and confirm the presence of the following grouping in the molecule IV.

The signals observed for the methyl groups are at 46.5, 52, 58, 76, 83, 91 and 96 cps. in bacogenin A$_1$. The assignment of these signals has been greatly facilitated by the work of Lehn and coworkers. The signal at 91 and 96 cps. are due to the methyl group of an isopropylidene group, where as the 83 cps. signal being an unusually high resonance, for one methyl group suggested a 1:3 interaction with an oxygen function and its assigned to methyl on C-14 stipulating a carbonyl group at position 16 in the molecule. This is supported by a similar observation reported recently in the case of one of the degradation
product of cucurbitacins. The signal at 76 cps is attributed to a methyl group on a carbon atom bearing an oxygen function analogous to the methyl on C\textsubscript{20} in the side chain of 20 hydroxy dammarene.

Since the above four methyl signals in bacogenin A\textsubscript{1} are not affected on acetylation (acetyl bacogenin A\textsubscript{1} gives signals at 51.5 (two) 53.5, 75.5, 82, 92 and 96 cps). They must be situated outside the sphere of influence of the acetylatable hydroxyl group in rings C, D and the side chain. Thus four possible positions in rings A and B are left for accommodating the three methyls (46.5, 52 and 58 cps) and a hydroxy methyl group (V). The presence of a methyl group only at C\textsubscript{14} and none at C\textsubscript{13} further confirmed that bacogenin A\textsubscript{1} belongs to the dammarene series and not to be the lanostane groups. Since the signals at 46.5 and 58 cps, in bacogenin A\textsubscript{1} undergoes a shift on acetylation to the 51, 53 cps region. The corresponding methyl group must be in proximity with the two hydroxyl group. The individual assignment of these signals cannot, however, be made with certainty because of absence of data on analogous compounds.

On the basis of physical and chemical data presented above, the partial structure for bacogenin A\textsubscript{1} can be presented by (V) in which the four position marked 'X' are meant to accommodate three methyl groups and one $-\text{CH}_2\text{CH}$ group.
FIG. 3 NMR SPECTRA OF ACETYL BACOGENIN A1
Further confirmation of position of various fractions and the fixation of hydroxy methyl group was obtained as follows. The acid dehydration of diacetyl bacogenin A\(_1\) led to a gum which could not be crystallized. However its chromatographic purification gave a product which showed UV maximum at 266 m\(\mu\) due to an \(\alpha\)-\(\beta\)-unsaturated keto group in the partial formula (VI) establishing the position of the keto group and tertiary hydroxyl groups beyond doubt.

Bacogenin A\(_1\) was treated with osmium tetraoxide and the subsequent oxidation of the diole with tetra-acetate gave acetone which was identified as acetone DNP. This confirmed the presence of isopropylidine group in the molecule. The precise placement of the quaternary hydroxyl methyl group on one of the four available position (marked as 'X' in (V)) was based on the following evidence – bacogenin A\(_1\) did not form any acetonide with acetone and anhydrous copper sulphate or sulphuric acid and did not undergo tritylation ruling out the gem-dimethyl position at C\(_4\).
The C₉ position is untenable on the basis of NMR Spectra because the C₁₄ methyl signal at 83 cps. remain unaffected on acetylation while it should have shifted if the hydroxyl methyl group was present at this position. C₁₀ is therefore, the only position available for the hydroxy methyl group. This is also supported by the hindered nature of this group as shown by its resistance to tritylation. The structure of bacogenin A₁ would thus be 3,10,20-trihydroxy-16-keto dammarene (24).

On the basis of data provided above the final structure of bacoside A as 3(α-L-arabinopyranosyl)-o-β-D-glucopyranoside, 10-20 dihydroxy 16 keto dammarene 24 (VII).
Bacogenin A$_2$

Bacogenin A$_2$ is an isomer of bacogenin A$_1$ differing in the configuration at C-20. It also gave positive Noller's test showing triterpenoids class of compound. It is soluble in all organic solvent and sparingly soluble in benzene and ether, $C_{30}H_{48}O_4$ (M$^+$ 472) mp. 220° contain an -OH (3350 cm$^{-1}$), a five membered ring $C=C$ (1750 cm$^{-1}$) and a $-C=O-H$ group (1665, 820 cm$^{-1}$). Its PMR spectrum exhibited signal for seven tertiary methyl $-CH_2-COCH-$, $-CHO-$, $-CH_2O-$ and Me $-C=CH$ groups. The relative disposition of the vinylic methyl and vinylic hydrogen as $-CH=C=Me$ was similar to that of bacogenin A$_1$ and was confirmed by spin decoupling. Further its PMR spectra in pyridine d$_5$ showed beside other signals a 24 AB quartet (J, 16 Hz) centered at 2.45 ppm and a 1H broad singlet at 2.47 ppm assignable to the methylene and methine protons respectively flanking the $C=O$ group thereby confirming the existence of a $-CH-CO-CH_2-$ grouping in the five membered ring.

After addition of trichloro acylisocynate the PMR spectrum of bacogenin A$_2$ exhibit two one hydrogen broad singlet of $-CO-NHCO-$proton (8.46 and 8.51 ppm) demonstrating the presence of only two hydroxyl group in the molecule. This was further confirmed by formation of bacogenin A$_2$ diacetate singlet at 2.0 and 2.06 ppm in PMR specimen. Moreover the
signals due to -CHO- and -CH₂O- now shifted downfield by 1 and 0.5 ppm respectively suggested that one of the -OH group was secondary and the other one primary. The mass spectrum of bacogenin A₂ displayed prominent peaks at m/e 472 (M⁺), 457 (m-15), 439 (M-15-18), 207 (ion a) and 189 (207-18). A very intense peak was observed at m/e 125 (base peak) due to the side chain (b)² which on further loss of H₂O and -CH₃ gave rise m/e 107 and 110 respectively. The mass spectrum of di-O-acetyl bacogenin A₂ also contained an intense peak at m/e 125 suggesting that the side chain of bacogenin A₂ did not carry any group.

In view of common molecular formula the functionalities of bacogenin A₁ and A₂ and their similar physico-chemical data bacogenin A₂ was considered to possess a gross structure similar to that of bacogenin A₁ but differing either in the configuration at C-17, C-20 in the disposition of the vinylic methyl group of the side chain.

![Diagram](image-url)
In case of C\textsubscript{17} \(\alpha\)-configuration, the side chain would be susceptible to base catalysed epimerisation because of activation by C-16 carbonyl group. Bacogenin A\textsubscript{2} was however recovered unchanged on alkali treatment indicating that of side chain possessed 17-\(\beta\) configuration, bacogenin A\textsubscript{2} has therefore been assigned structure as -

\[
\begin{align*}
\text{Bacogenin A}_2 \quad \text{HO} \\
\text{CH}_2\text{OH} \\
\end{align*}
\]

Stereochemistry of Bacogenin A\textsubscript{1} and A\textsubscript{2}

The treatment of di-o-acetyl bacogenin A\textsubscript{1} with RuO\textsubscript{4} led to the formation of 12 diole instead of the usual secondary dioxyo product. The diole could not be cleaved by periodate probably because of its highly hindered nature. Di-o-acetyl bacogenin A\textsubscript{1} was therefore treated with a solution of RuO\textsubscript{4} in presence of NaI0\textsubscript{4}. The reaction mixture showed two major spots (TLC) designated as UA\textsubscript{1} and LA\textsubscript{1} which were separated by chromatography. Substance LA\textsubscript{1} \(\text{C}_{34}\text{H}_{52}\text{O}_{9}\) was soluble in alkali indicating its acidic nature which was evident in its IR Spectrum by a broad \(-\text{OH}\) bond extending from 2350-3450 cm\textsuperscript{-1} and composite \(\text{C}=\text{O}\) bond at 1750 cm\textsuperscript{-1}. 
The product LA₁ furnished a methyl ester (methyl LA₁) 
\( \text{C}_{35}\text{H}_{54}\text{O}_9 \). Its IR Spectrum did not show any absorption in the 
-\( \text{OH} \) region. The PMR spectrum of methyl LA₁ manifested the 
presence of four tertiary C-\( \text{CH}_3 \) groups –

![Chemical structure](image)

almost in the same magnetic environment as in di-\( \text{o} \)-acetyl 
bacogenin A₁ (6H, S at .883 ppm and 3H each S at .966 and 
1.166 ppm) besides there other tertiary C-\( \text{CH}_3 \) groups (6H, S 
at 1.350 and 3H, S at 1.425 ppm), one C-CO-\( \text{CH}_3 \) (3H, S at 
2.290 ppm), one –COOCH₃, one primary and one secondary acetox: 
groups. The absence of vinylic proton and vinylic methyl 
signal clearly demonstrated that the double bond in the
side chain of di-o-acetyl bacogenin A₁ had cleaved resulting in the generation of -COCH₃ and -COOH group. The structure of the side chain of methyl LA₁ could, then be represented either by II or III depending on the disposition of vinlylic -CH₃ in the side chain of bacogenin A₁.

A choice between II and III could be made by the mass fragmentation pattern of methyl LA₁. Although the molecular ion peak was not visible, a prominent peaks at m/e 575 was due to fragment (a) formed by the loss of -CH₃CO (M⁺-43). The peak at m/e 475 (fragment b) was due to loss of CH₂=CH₃COOCH₃ from fragment a by a hydride transfer (m at m/e 392.4). The fragment b then lost 42 (CH₂=C=O, m* at m/e 394.7) at 60 (CH₃COOH, m* at m/e 321.3) mass units in a sequence giving rise to peaks at m/e 433 (c) and 373 (d) respectively. The peaks at m/e 353 (m* at m/e 337.8) was due to the loss of H₂O from m/e 373 (scheme I).

Thus, the elimination of -CH₃CO radical followed by natural fragment, CH₃=C(CH₃)-COOCH₃ from the molecular ion demonstrated unequivocally that methyl LA₁ possessed the side chain II. Consequently C-22 was established as the position of the vinlylic methyl group in bacogenin A₁ which could now be represented as IA. The absolute configuration at C-20 has been deduced later.
The product $\text{UA}_1 \ C_{34}H_{52}O_8 \ (M^+ \ 588)$ contained two additional oxygen atoms incorporated during the reaction. Its IR Spectrum indicated the presence of a $\text{-OH}$ group ($3500 \ \text{cm}^{-1}$) and an additional $\text{-CO}$ group in a five membered ring ($1762 \ \text{cm}^{-1}$). The hydroxyl group was tertiary in nature as it could not be acetylated and the PMR showed the absence of any carbinol proton ($\delta$). The PMR spectrum also locked the vinylic hydrogen and methyl signals, instead there was a singlet at 1.46 ppm for a methyl group situated on an $\alpha$-ketol which was confirmed by the identity (TLC, IR, NMR, MS) of $\text{UA}_1$ and $\text{CrO}_3$ oxidation product of the bacogenin $\text{A}_1$ diole mentioned earlier. The product $\text{UA}_1$ would, therefore have the structure IV. It may be mention that the oxidation of diole under the conditions specified in the experimental, also yielded a small amount of another product which was formed to be identical with $\text{LA}_1$.

Di-o-acetyl bacogenin $\text{A}_2$ was oxidised with $\text{RuO}_4$-$\text{NaIO}_4$ under the same condition as employed for the oxidation of di-o-acetyl bacogenin $\text{A}_1$ and two analogous substance $\text{UA}_2$ and $\text{LA}_2$ were isolated. The substance $\text{LA}_2$ was alkali soluble and its IR data indicated the presence of a $\text{-COOH}$ group. On treatment with $\text{CH}_2\text{N}_2$, it furnished a methyl ester (methyl $\text{LA}_2$) $C_{35}H_{54}O_9$. The PMR spectrum of methyl $\text{LA}_2$ exhibited the presence of seven tertiary $\text{C-CH}_3$ (6H, $\delta$, at .883 and 3H each at .941, 1.138, 1.28, 1.50 and 1.58 ppm) two $\text{-COCH}_3$ groups.
a -COOCH₃, a CH₂-OAc and a -CHOAc. The LA₂ and methyl LA₁ were different substances evident from their optical rotations and differences in the position of the non-skeletal methyls belonging to side chain.

Its mass spectrum was found to be exactly similar to that of methyl LA₁ displaying peaks at m/e 575 (m⁺-43), 475 (M⁺-43-100, m⁺ at m/e 392.4), 433 (475-42 m⁺ at m/e 394.7), 373 (433-60 m⁺ at m/e 321.3). This demonstrated that the vinylic methyl in bacogenin A₂ occupied the same position i.e. C-22 as in case of bacogenin A₁. Bacogenin A₂ and bacogenin A₁ were therefore, isomers differing in the configuration at C-20.

The substance UA₂ C₃₄H₅₂O₈ (M⁺ 588) showed in its IR spectrum the presence of a -OH and a C=O group in a five-membered ring. Its PMR and mass spectra were also very similar to those of UA₁. These data directly indicated the LA₂ and UA₂ were isomers of LA₁ and LA₂ could be represented as V and VI respectively.
In inspection of Dreiding model of 20S epimer of the methyl ester showed that the side chain would adopt the (+) anti-periplaner configuration VII associated with the least steric interaction. It could be seen that in this confirmation the 16. -C=O group was situated sufficiently away from the gem dimethyls on C-25 and was not expected to experiences a little deshielding. This situation in fact, was observed in the case of methyl LA₁. Thus its PMR spectrum showed a 6H singlet at 1.350 ppm and 3H singlet at a slightly lower field 1.425 ppm assignable to C₂₅ gem methyl and C₂₀ methyl respectively. The configuration of methyl LA₁ at C-20 and hence that of bacogenin A₁ was established as 'S'. The absolute structure of bacogenin A₁ could be represented as 3β, 30 dihydroxy 20(s)- 25 epoxy 22 methyl 24 nordammer 22 en -16 one (IX). The X-ray crystallographic studies on bacogenin
A₁ dibromoacetate have also confirmed the above molecular structure and absolute configuration of bacogenin A₁.

The most preferred configuration that the side chain would acquire in the Dreiding model of the 20R epimer of the methyl ester was on the other hand, found to be (+) synctinal (VIII). Consequently the C₂₀-CH₃ being held above the 16-C=O group, would be shielded conversely the C₂₅ gem-dimethyl would be in the plane of 16-C=O group and would be deshielded. These observations were in agreement with the chemical shift of C-2₅ and C-2₀ methyl group in the PMR spectrum of methyl LA₂ which showed a 3H singlet at 1.28 ppm for C₂₀-CH₃ and two downfield singlets 3H each, at 1.5 and 1.55 ppm for C₃₅ gem dimethyls. Thus the configuration of methyl LA₂ at C₂₀ was confirmed as R, thus the absolute structure of bacogenin A₂ would be represented by (X).
Bacogenin A₃

Bacogenin A₃ C₃₀H₄₆O₃ (M⁺ 454) IR absorption at 3344 cm⁻¹ (OH) exhibits NMR signals for four tertiary C-Me (0.78, 0.84, 0.98, 1.03 ppm), three vinyllic C-Me (6H d, J, 1.5 Hz, 1.53 ppm and 3H, d, J, 1.5 Hz, 1.76 ppm), two -CH₂O (broad S, 4.1 ppm), one -CHO (m, 3.21 ppm) and vinyllic H (m, 5.43 ppm). Its UV spectrum indicated the presence of a diene chromophore 230 nm E 9630.

Bacogenin A₃ furnished a mono acetate (lb) C₃₂H₄₈O₄ which showed IR absorption at 1730, 1260 cm⁻¹ (-CCO-Me). The absence of -OH group in the IR spectrum and the presence of a singlet due to acetoxyethyl at 2.05 ppm with a concurrent shift of the carbanolic proton signal to 4.48 ppm (from 3.21 ppm) in the NMR spectrum indicated the presence of the only one secondary -OH group in the molecule. The two inert oxygen were therefore ethereal in nature and each must be linked to a methylene group which appeared responsible for a 4H broad singlet at 4.1 ppm.
The ozonolysis of (1b) in the presence of pyrene led to a selective cleavage of one of the double bonds to give a trinoraldehyde (3) \( \text{C}_{29}\text{H}_{42}\text{O}_{5} \) (\( M^+ \) 470) conjugated to with a tetrasubstituted double bond (1670, 1635 cm\(^{-1}\)) 243 nm (\( \Sigma 1150 \)). Its NMR spectrum showed a sharp singlet at 9.96 ppm for an aldehydic proton which demonstrated the presence of a 
\(-\text{C=CH=CH=C} \) (Me) group in bacogenin A\(_3\).

**COMPOUND (1b)**

On catalytic hydrogenation yielded a hexahydro product (4a) \( \text{C}_{32}\text{H}_{54}\text{O}_{4} \) (\( M^+ \) 502) which showed -OH absorption
(3350 cm\(^{-1}\)) in the IR but was transparant in the UV. In the NMR spectrum only one of the \(-\text{CH}_2\text{O}\)-group manifested itself by an AB quartet (J 12 Hz) at 3.96 ppm which shifted to 4.43 ppm in its acetylated product (4b) with the appearance of an additional acetoxo methyl shift at 1.96 ppm. The hexahydro product (4a) therefore contained a \(-\text{CH}_2\text{OH}\) group on a fully substituted carbon.

The saponification of compound (4b) furnished product (4c) \(\text{C}_{30}\text{H}_{52}\text{O}_3\) (\(M^+\) 460) whose IR spectrum displayed the presence of \(-\text{OH}\) (3344 cm\(^{-1}\)) and a cyclopentanone ring (1727 cm\(^{-1}\)) in the molecule. The NMR spectrum was devoid of any vinylic methyl signal but showed four tertiary-\(-\text{C-Me}\) (0.80, 0.90, 1.00, 1.10 ppm), \(-\text{CH}_2\text{-CO}\) (ABq 16 Hz 2.18 ppm), \(-\text{CH}_2\text{-OH}\) (m, 3.24 ppm), \(-\text{CH}_2\text{OH}\) (ABq 12 Hz 4.0 ppm). The position and the splitting of the NMR signal due to four tertiary-\(-\text{C-Me}-\text{CHOH}\) and \(-\text{CH}_2\text{OH}\) were very similar to those of possess the same skeleton (dammarene type) with identical placement of \(-\text{CHOH}, -\text{CH}_2\text{OH}, -\text{C=O}\) group and the side chain at C-3 (\(\beta\)), C-18 (\(\alpha\)), C-16 and C-17 (\(\beta\)) respectively. The AB quartet (J 16 Hz) centered at 2.18 ppm could thus be assigned to the C-15 methylene proton.

Further the mass spectrum of (4c) displayed as \(M^+\) peak at m/e 460 beside other prominent peak at m/e 442 (\(M-\text{H}_2\text{O}\)), 429 (\(M-\text{CH}_2\text{OH}\)), 411 (\(M-\text{H}_2\text{O}\)), 207 (ion a), 187 (ion b) and 113 (side chain). The abundant ion m/e Lafferly
rearrangement confirmed the relative position of the side chain and the C=O group. The formation of fragment ion a and b proved that primary alcohol and could be accommodated only at C-18 in the dammarane skeleton.

The generation of the carbonyl group, -CH₂OH and a secondary methyl in the hexahydro product (4a) clearly demonstrated the presence of a cyclic ketal in a bacogenin A₃ which opened up hydrogenation with the concurrent hydrolysis of a vinylic -CH₂-O-group. The other three secondary methyl group must be located on the tetrasubstituted double bond of the diene system. Since it remained intact after azonolysis of bacogenin A₃ acetate (1b) and appeared as a singlet at 4.15 ppm in the NMR of the aldehyde (3). Thus the hexahydro product (4a) carried the side chain -CH = C - CH₂ - CH (Me)₂. The reconstruction of the diene side chain and a ketal from the hexa-hydro product (4c) would lead to two structure (1a) and (2) for bacogenin A₃. Structure (2) was however ruled out on the ground that in none of the bacogenin does C-21 bear an oxygen function. Bacogenin A₃ would be therefore reported by structure (1a).
Bacogenin A₄

Bacogenin A₄ mp 175°C C₃₀H₄₁O₃ (M⁺ 544) was found to be the major component in aglycone mixture. It showed λ max 269, 279, 291 nm and formed a mono acetate mp 212°C C₃₂H₄₉O₄. It has been identified as ebeline lactone (l) from its chemical and physiological data and finally by the direct comparison (TLC, mmp, PMR and IR) of mono-o-acetyl bacogenin A₄ with authentic sample.

Bacoside A did not show any UV absorption demonstrating that the triene system of ebeline lactone was absent in the genuine sapogenin and was generated under acedic condition of hydrolysis. The IR spectrum of bacoside A was also devoid of carbonyl absorption and therefore it must carry a latent carbonyl group probably in the form of a ketal as in case of cimiginol. A more interesting fact was that while ebeline lactone has a normal disposition of isoprene units in its side chain bacogenin A₁ contained a rearranged side chain and both of there must have arise from common precursor the presence of a cyclopropane ring in the side chain of the genuine precursor of the ebeline lactone. In view of these fact a tentative structure was being proposed as a working basis for future studies.
CHARACTERISATION OF COMPOUND B

Bacoside B is obtained as colourless needles from methanol mp 203°C $[\alpha]_D = +8^\circ$ (80% alcohol). It is sparingly soluble in ethanol, methanol and water and analysed for $C_{47}H_{76}O_{19}$. It could not be hydrolysed with emulsion or dilute ethanolic sodium hydroxide solution. However under similar condition to bacoside A hydrolysis was effected with dilute methanolic sulphuric acid and the neutral aqueous hydrolysate shows two spots $R_f$ 0.20 and 0.24 on paper chromatography in butanol: acetic acid: water (4:1:5) and development with aniline hydrogen phthalate. The characterisation of these constituents units of the carbohydrate chain as glucose and arabinose was done by paper chromatographic separation comparison in various system.

The total sugar in the molecule of bacoside B were estimated iodometrically and found to be 35.1%. This was confirmed gravimetrically by the reduction of Fehling solution as cuprous oxide which gave a value of 34.7%. The remaining 65% of the molecule was accounted for as the aglycone content.
The ratio of two sugar in the molecule were determined as in bacoside A. The glucose and arabinose are present in equimolar ratio showing identical sugar constitution.

The aglycone fraction showed many spots on thin layer chromatogram giving the similar sapogenins as A₁, A₂, A₃ and A₄ reminiscent of the behaviour of analogous fraction obtained by the hydrolysis of bacoside A. The total aglycone fraction (3 gm) obtained by chloroform extraction of acid hydrolysate of bacoside B (5 gm) was chromatographed over silica gel G.

A mixture of sapogenins A₁, A₂, A₃ and A₄ were obtained which has the same gross structure proposed for the sapogenins of bacoside A.

It would thus seem clear that the bacoside A and B are identical in respect of their respective carbohydrate and the aglycone moieties and therefore bacoside B is dextro-rotatory and bacoside A is laevorotatory and the homolytic activity of the former is twice that of the later. This may be explained on the assumption that configuration of the carbohydrate chain is different in the two glucoside.

The characterisation of bacoside B and its hydrolysed product i.e. glucone and aglucone is as same as of bacoside A, so the details studies of these were identical with the bacoside A.
CHARACTERISATION OF COMPOUND C

It is obtained as a crystalline solid form alcoholic extract mp 166°C. It was soluble in water and insoluble in rest of the solvent.

It forms well defined crystalline derivative of acetate mp 124°C.

No depression was observed in mixed mp of the derivative of substance with respective derivative of an authentic specimen of D-mannitol.

It gives all the colour reaction for the hydroxyl group.

On the basis of melting point, specific rotation, colour test, acetate, it was concluded that compound C, is D-mannitol,

\[ \text{D-mannitol} \]

CHARACTERISATION OF COMPOUND D

It was obtained in flask from the petroleum ether
It was obtained in flask from the petroleum ether (60-80) extract, which after crystallisation from methanol was obtained as silky needles mp. 315°C. Compound was insoluble in common organic solvent other than ethanol and methanol.

It forms well defined crystalline derivative such as acetate mp. 276°C, benzoate mp. 292.5°C and methyl ester mp. 224°C depression.

No was observed in mixed melting point of the derivative of substance with respective derivative of an authentic specimen of betulinic acid.

It did not show any absorption in the UV region. The IR spectrum of the acid indicate a close resemblance with betulinic acid, but some differences are also observed. In addition to band at 1643 and 882 cm\(^{-1}\) similar to those of betulinic acid another band at 1652 cm\(^{-1}\) is obtained. For a further comparison the IR spectrum of the ester of both the acid being studied. On reduction with lithium aluminium hydride betulin was obtained, compared with the authentic specimen confirmed its identity.

On the basis of melting point, specific rotation, colour test, acetate, benzoate and methyl derivatives it was concluded that compound D is betulinic acid.
Fig. 4. I.R. Curve of Betulin
CHARACTERISATION OF COMPOUND E

It was isolated from the light petrol soluble fraction as chromatographically homogeneous, crystalline needles, mp. 170°C $[\alpha]_D = -47^\circ$ (chloroform).

On the basis of carbon-hydrogen analysis and molecular weight determination the molecular formula was calculated as $C_{29}H_{48}O$. The compound is soluble in benzene and chloroform but sparingly soluble in methanol. It gave yellow colour changing to red (solkowski reaction)$^6$.

The above colour reaction is specific for sterols. It formed monoacetate mp. 141°C and benzoate 160°C and digitonide mp. 100°C. It was identified as stigmasterol by co-paper chromatography with an authentic sample and mixed melting point was also undepressed.

Stigmasterol is one of the most commonly occurring sterol in nature.

Stigmasterol
CHARACTERISATION OF COMPOUND F

The ether filtrate on elution with benzene: chloroform (1:2) gave a non-terpenoids compound F which on repeated crystallisation, from chloroform methanol produced colourless plates mp. 136-137°C (α)\textsubscript{D}^25 = -37° (chloroform).

On basis of carbon-hydrogen analysis and molecular weight determination the molecular formula was calculated as C\textsubscript{29}H\textsubscript{50}O.

The compound is soluble in benzene and chloroform but sparingly soluble in methanol.

It responded to Liebermann-Burchard\textsuperscript{7} and Solkowski\textsuperscript{5} and Rosenheim\textsuperscript{8} colour tests which are specific for the steroidal structure. It developed pale yellow colouration with tetrannitromethane and decolourised bromine in carbon tetrachloride, indicating the presence of unsaturation in the molecule. This formed well defined crystalline derivative such as acetate mp. 127°C, benzoate mp. 144°C and a digitonide mp. 227°C.

No depression was observed in mixed melting point of the derivative of this substance with respective derivative of an authentic specimen of β-sitosterol. The substance was oxidised with aluminium tertiary butaoxide in acetone solution and a ketone mp. 87°C was obtained. 2,4-dinitrophenyl hydrazone derivative of the ketone was prepared. Its melting point and mixed melting with 2,4-dinitrophenyl-
hydrazone derivative and β-sitos-4-enone was 250°C. IR spectrum comparison with an authentic specimen confirmed its identity. On the basis of melting point, specific rotation colour test, acetate, benzoate and degitonides formation, oxidation to a ketone, it was concluded that the compound F is β-sitosterol.

CHARACTERISATION OF COMPOUND G

It was isolated as cream coloured, hygroscopic semi crystalline, chromatographically single compound $[\alpha]_D^{20} + 9.8$ (acetone). It gave blue precipitate with FeCl$_3$ and positive Molisch test$^{10}$ indicating it to be a polyphenol glucoside. However positive test with aniline hydrogen phthalate reagent$^{11-12}$ showed the presence of a potential aldehyde group in the sugar moiety. Alkali as well as acid hydrolysis of the compound gave glucose and gallic acid. Therefore it could be a galloylester of glucose. The quantitative hydrolysis indicated the presence of six galloyl groups
FIG. 5. I.R. CURVE OF β-SITOSTEROL
per molecule of glucose. As the compound was highly hygroscopic, despite all precaution it analysed every time for different member of molecules of water of crystallisation. Molecular weight determination also could not be done by usual methods. Its acetate was prepared, which being not so hygroscopic could be analysed. Its analysis agreed with that requires for hexagalloyl glucose.

In order to get more information methylation with diazomethane and subsequent hydrolysis of gallotannin were carried out this gave a mixture of 3,4,5-tri-o-methyl and 3,4-di-o-methyl gallic acid which were identified by paper chromatography and mixed melting point with respective authentic samples. The detection of 3,4-di-o-methyl gallic acid in the hydrolysate indicated the presence of depside links in the molecule. The intensity of the two spot of these on a descending strip chromatogram, sprayed with bromophenol blue was compared with the intensity of the spots obtained by running the artificial mixture of authentic 3,4,5-tri-o-methyl and 3,4-di-o-methyl gallic acid by weight in different molecular proportion. The chromatogram of the hydrolysate agreed well with (2:1) molar mixture of tri-o-methyl and di-o-methyl gallic acid which supported the conclusion that four ester and two depside linkages may be present in the molecule. The presence of four ester linkage in the molecule of the tannin would mean that one hydroxyl of glucose moiety
is free. The gallotannin as well as its methylated products with diazomethane on paper chromatogram gave a purplish brown spot when sprayed with aniline hydrogen pthalate reagent.\(^{12-13}\) It is characteristic test for a free potential aldehyde group therefore the position of ester linkage could be 2, 3, 4 and 6 provided glucose is present in its pyranose form. This test was utilised by Hawarth et al.\(^9\) established the position of ester linkages in tetra galloyl glucose as 2, 3, 4 and 6. The tannin in the present case after complete methylation with diazo methane reduce Fehling solution which further confirmed the presence of the free reducing group.

The position of the two depside links had to be decided. These could be present in chain i.e. as a trigalloyl chain or a two digalloyl chains attached to glucose core at two different position. It could be decided by subjecting the tannin to methanolysis i.e. it was treated with aqueous methanol at pH 5.6. It is reported\(^16\) that depside linkages particularly those ortho to phenolic group are prone to attack by methanol and undergo methanolysis with 90% methanol at pH 5.6 at 31°C yielding the methyl ester e.g. methyl 3-O-benzyol proto catachuate (I) yield methyl benzoate (III) and methyl protocatachuate (II). Similarly results were obtained when depside having phenolic hydroxylic in ortho position to the depside linkages. On the other hand the depside not having any phenolic group ortho to the depside
linkage and ester of gallic acid e.g., tri-o-galloyl glycerol
and β-pentagalloyl-D-glucose were unaffected by methanol. It
has been pointed out by Haworth et al.\textsuperscript{16} that alcoholysis
take place by both methanol and ethanol although it is more
rapid with the former. This reaction may be called tran-
esterification process. It is assumed to be taking place by
a combination of different mechanism.

During extraction with ethanol pH of the ethanolic extracts
being 5.5 ethanolysis of (IV) may produce ethyl gallate (V)
as shown below -
On controlled methanolysis the methylated, tannin gave tri-o-methyl gallate and di-o-methyl gallate indicating the presence of two depside linkages in the same chain i.e. a trigalloyl chain is present. However m-digallate could not be isolated. This may be due to the reason that if at all formed it probably gets quickly hydrolysed to tri-o-methyl gallate and di-o-methyl gallate.

If 2 molar digalloyl units were linked at two different positions on the glucose core only tri-o-methyl gallate would have been formed and not dimethyl gallate during methanolysis.

From the above observations it could be concluded that four galloyl units are linked through four ester linkages at 2, 3, 4 and 6 positions of glucose molecule and at anyone of the position a trigalloyl chain is present, having two gallic acid units linked through depside linkage, although the exact position of the trigalloyl chain could not be decided, but the stereochemistry of the molecule as well as biogenesis of this class of compound favours position six. In all the natural occurring gallotannin so far reported the longest chain of gallic acid residue is always attached at position six. The low optical rotation (α)_D^20 + 9.8 (acetone) of gallotannin suggested that probably glucose is present here in β form. Thus structure (VII) could be proposed for the tannin.
Its IR spectrum showed a definite ester bond at 1720 cm\(^{-1}\). This ester bond was slightly notched as if having two different frequencies. This may account for the presence of ester and depside linkages which could be present close together.

The NMR spectrum of methyl ether acetate of this tannin agreed with the proposed structure. \(\delta\) 2.05 corresponding to 3 acetyl proton (acetyl at C-1 of glucose) and 6.3.95 corresponding to methoxyl proton integrating for 5,4 proton. As there are 1,6 methoxy group in the molecule

(VII)

Gallotannin
the remaining 6 proton could be attributed to the sugar proton viz., at C-2, C-3, C-4, C-5 one each and two proton at C-6. Signal for sugar proton and methoxyl proton appears to have been merged. Twelve aromatic protons belonging to six gallic acid units show a signal at 6 7.3. The signal proton at C-1 of glucose shows as a broad signal at 65.0.
NATURALLY OCCURRING TANNINS

Tannins are polyphenols widely distributed in the vegetable kingdom. The property of astringency in food and beverages is commonly ascribed to the presence of tannins. Their ability to combine with protein is the basis of the process known as vegetable tanning by which the animal skin is converted into leather. In addition to this, these have several other industrial and technological applications such as in the manufacturing of inks, and plastics, the preservation of fish nets, in oil well drilling and as moderant in dyeing.

According to Freudenberg\textsuperscript{29} the vegetable tannins can be divided into two main groups which arise from their behaviour towards hydrolytic agents particularly acids.

(i) THE CONDENSED OR NONHYDROLYSABLE TANNINS

These undergo progressive polymerisation under the action of acids to yield the amorphous tannins reds.

(ii) THE HYDROLYSABLE TANNINS

These undergo hydrolysis with acid, alkalies or enzyme and yield carbohydrate usually glucose or related polyhydric alcohol and one or more phenolic acids. The hydrolysable tannin have been classified as -
(a) Gallotannins
(b) Ellagitannins

Gallotannin on hydrolysis give gallic acid and glucose while ellagitannin gives ellagic acid along with gallic acid or other related phenols. But the above classification seems to be unsatisfactory now as some tannins like tara tannins donot gives any carbohydrates on hydrolysis but instead have quinic acid acting as the basic core.

The hydrolysable tannins are often isolated as amorphous but some time as microcrystalline solids. Their homogenity is usually ascertained chromatographically or by taking optical rotation. However, the homogenity of tannins is still a debatable point. The method of isolation, difficulties in purifications and the chemistry in general of these hydrolysable tannin has been reviewed by Haslam. A large number of galloyl esters of glucose varying in complexity from the simple mono and diesters to (-) epicatechin and (-) epigallocatechin gallates, Hamameli and Acertannin to the more complicated gallotannin, Chinese, Sumach, Tara and Turkish tannin are known to occur in nature. These naturally occurring gallotannins have been tabulated as on page (70).

According to White only those polyphenolic molecules which have molecular weight in the range of 500-3000 have tanning action e.g. the gallotannins, Chinese, Sumach, Tara, and Turkish tannins are tannins in the real sense of term.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Tannins</th>
<th>Structure</th>
<th>Sources</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>-D-glucogallin</td>
<td>1-O-galloyl-D-glucose</td>
<td>Roots (Chinese Rhubarb, E. officinale)</td>
<td>Gilson, E. 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Myrobalanas, Eucalyptus</td>
<td>Hathway 35, Hillis, Carle 36</td>
</tr>
<tr>
<td>2.</td>
<td>Epicatechin gallate</td>
<td>3-gallo ester of epicatechin</td>
<td>Camellia sinensis</td>
<td>Robert 37, Tsujimura 38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bradford 37, Tsujimura 38</td>
</tr>
<tr>
<td>3.</td>
<td>Epigallo-Catechin</td>
<td>3-galloyl quinic acid</td>
<td>Camellia sinensis</td>
<td>Roberts, Myers 40</td>
</tr>
<tr>
<td></td>
<td>gallate</td>
<td></td>
<td>(leaves)</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Theogallin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Hamamelis Tannins</td>
<td>2-5 di-o-galloyl-D-hamamelose</td>
<td>Hamamelis virg nicastenea sativa (Bark)</td>
<td>Van Gruther 41, Mayer and Kunz 42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-penta-o-galloyl-D-glucose</td>
<td>Terminalis chebula (Myrobalanas)</td>
<td>Russel, A. 43, Tebbens, W.G.</td>
</tr>
<tr>
<td>6.</td>
<td>Acer tannins</td>
<td>-3,6 di-o-galloyl-1-5 anhydro-D-glucitol</td>
<td>Leaves of Korean Maple (Acer gingale)</td>
<td>Perkin and Vyeda 44</td>
</tr>
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<td></td>
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</tr>
<tr>
<td>7(a)</td>
<td>Chinese gallo-tannins(a)</td>
<td>Hepta-nona-o-galloyl D-glucose</td>
<td>Leaves of Rhus armitage et al. 45</td>
<td></td>
</tr>
<tr>
<td>7(b)</td>
<td>Dhava gallo-tannin(b)</td>
<td>-do-</td>
<td>Anogeissus latifolia (leaves) Reddy and Nayudamma 46</td>
<td></td>
</tr>
<tr>
<td>7(c)</td>
<td>Sumach gallo-tannins(c)</td>
<td>-do-</td>
<td>Rhus typhinia and Haslam, et al. 47</td>
<td>Rhus cariaria</td>
</tr>
<tr>
<td>8.</td>
<td>Tara gallotannins</td>
<td>Tetra-penta-o-galloyl quinic acid</td>
<td>Caesalpinia spinosa (fruit pods) Haslam, Hawarth 47</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Turkish gallo-tannins</td>
<td>Hexa-hepta-o-galloyl -D-glucose</td>
<td>Quercus infectoria Haslam et al. 47</td>
<td></td>
</tr>
</tbody>
</table>
The earliest sources known for gallotannin were Aleppo and Chinese gallotannins. Many workers isolated and studied gallotannins from the sources from time to time. Alcohols, water and ether mixture were used by almost all of these early workers for extraction of the gallotannins hence their preparations were impure containing gallic acid its esters and mineral salts along with gallotannins. Fischer and Freudenberg claimed to have isolated pure Chinese gallotannins by removing free gallic acid but it was found to be mixture by Iljin its results were further confirmed by Karrer and his coworkers who reported that gallotannin are mixture of different galloyl glucose.

Fischer and Freudenberg concluded, based on the proportion of gallic acid and glucose obtained by hydrolysis of tannin itself and the yields of 3-4-5-tri-o-methyl and 3-4-di-o-methyl gallic acid obtained by alkaline hydrolysis of a methylated tannins that Chinese gallotannins was a mixture of isomers and closely related compounds with an average composition corresponding to \( \beta \)-penta-m-digalloyl glucose (9) \( (R'=R''=A) \).

Fischer however envisaged the possibility of the presence of tri or even tetra galloyl chain in the Chinese gallotannin and Freudenberg pointed out that many arrangements varying from penta-m-digalloyl glucose to a compound (9) where \( R'=B \) and \( R''=C \) will agree with the experimental
observation. White et al. \(^54\) claimed carbohydrate core of
gallotannin was a tri saccharides and not glucose. Grassman
and coworker \(^55\) also reported Stragshorm Sumach tannin as a
polygalloylated tetra saccharides. But Hawarth et al. in
their important work on gallotannin have proved beyond doubt
that the carbohydrate core of the gallotannin is only glucose
and not any other polysaccharides. They hydrolysed the Chinese
and Sumach tannins by means of specific galloyl esterase \(^56\)
isolated by ion exchange chromatography of the enzyme tannase
and obtained only glucose and gallic acid. The isolation of
galloyl esterase was a significant advances in gallotannin
chemistry as the enzyme had no carbohydrate activity. By
using it the claim of earlier workers that tannins contain
polysaccharides were disappointed. White and Endres \(^57\) also
accepted this view lateron.

Introduction of a novel reaction i.e. methanolysis \(^58\),
the structural elucidation of these tannins in a significant
advance. In this reaction galloyl groups bound depsidically
in gallotannins are cleaved from the core of tannin by the
action of methanol at neutral pH. Methanolysis of Chinese
and Sumach (7b) gallotannin by Hawarth's et al. \(^59\) yielded
methyl gallate \(\beta\)-penta-o-galloyl-D-glucose (10a) and
\(\beta\)-2,3,4,6-tetra-o-galloyl-D-glucose (10b). The formation of
(10b) in small amount during methanolysis was explained by
these authors as resulting from partial breakdown of the
tannin before or during its isolation.

The 1:1 proportion of 3,4,5-tri-o-methyl and 3,4-di-o-methyl gallic acids obtained by complete methylation and hydrolysis of these tannins led these authors to conclude that the number of ester linkage is equal to that of depside linkage in the molecule. The presence of methyl-digallate, detected by their paper chromatography at an intermediate stage during methanolysis and isolation of m-tri gallic acid during acid hydrolysis of Chinese gallotannin (7a) further confirmed the presence of chain of at least three galloyl groups in the tannin, thus Hawarth and coworkers concluded that these gallotannin contain a β-penta-o-galloyl glucose core to which three or four additional galloyl groups are attached by depside linkages seems to be in confirmation with Fischer's proposal of their structure made about sixty years back.

However the desposition of depsidically linked galloyl groups on the basic cores of these gallotannin remain an enigmatic point. The distribution may be random and this would support the view that attempts at fractination of the gallotannin leads to a conclusion that these are mixture of an extremely complex nature.

The improved modern technique of purification like column chromatography, counter current distribution and
electrophoresis have made the isolation of pure tannins fairly easy now. With these improved techniques more light is expected to be thrown on the rather complicated structure of these tannins.
\[ R^2 = R^3 = R^4 = R^6 = H \]

1. \[ R^1 = \begin{array}{c}
\text{C} \\
\text{OH} \\
\text{OH} \\
\text{OH}
\end{array} \]

2. \[ \text{with structures as shown} \]

3. \[ \text{with structures as shown} \]

4. \[ R^1 = \begin{array}{c}
\text{C} \\
\text{OH} \\
\text{OH} \\
\text{OH}
\end{array} \]

5. \[ R^1 = \begin{array}{c}
\text{C} \\
\text{OH} \\
\text{OH} \\
\text{OH}
\end{array} \]

6. \[ R^1 = \begin{array}{c}
\text{C} \\
\text{OH} \\
\text{OH} \\
\text{OH}
\end{array} \]
(a) $R = \begin{array}{c} \text{OH} \\ \text{CH}_2 \end{array}$  \text{7 or 9}

(b) $R = \begin{array}{c} \text{OH} \\ \text{CH}_2 \end{array}$  \text{6 or 7}

(7)

(8)

(9A)

(9B)
EXPERIMENTAL

The recorded melting points are uncorrected and the following instruments, abbreviations etc. have been used. The IR spectra were recorded on Perkin-Elmer Infra cord 137. NMR spectra on a varian A-COD spectrometer using tetramethyl silane as a reference standard. The NMR spectra were taken in CDCl₃, unless otherwise stated. Chemical shift values are expressed in J scale and NMR spectra recorded in presence of tri-chloroacetyl-isocyanate. Mass spectra on Hitachi RMU-6E single focusing spectrometer Whatmann no. 1 filter paper was used for paper chromatography (Pchr) and silica gel G for thin layer chromatography (TLC). 1% solution of Aneline hydrogen phthalate, alcoholic FeCl₃ has been used as a general spray reagent.

(A) ISOLATION OF THE CONSTITUENTS

Freshly collected and air dried, powdered whole plant (6 Kg) was moistened with water and extracted with alcohol. The alcohol extract was concentrated at 50°C under reduced pressure and left in the cold when a crystal deposited. was obtained which was filtered (1.5 gm), mp. 160°C. This was recrystallized from dilute methanol a colourless needles mp 166°C (C).

The filtrate was diluted to 60% ethanol (X) and repeatedly partitioned with benzene (Y). The ethanol fraction
was treated with excess of lead acetate (500 gm), the lead salt was filtered and the residue lead was removed from the filtrate with H₂S. The pH of the final filtrate was adjusted to 6.3 with sodium carbonate and the solution was concentrated at 50°C in vacuo. During concentration viscous slimy material precipitated out which was separated from supernatant (Z) by centrifugation. The slimy material was dissolved in alcohol and fractionally precipitated with ether and petroleum ether repeatedly leading to a separation brown resinous material.

The alcohol, ether, petroleum ether solution was concentrated in vacuo and residue was macerated with acetone and filtered. The acetone insoluble powder was partitioned between butanol and water. During concentration of the butanol solution in vacuo a precipitate settled down which was (F-I) (50 gm), mp 225°C (decomp.). The filtrate was again concentrated and precipitated with acetone and ether which yielded a powder (F-II) (15.6 gm), mp. 246-8°C (decomp.). The mother liquor was concentrated to dryness when a brown powder (F-III) (21 gm), mp. 220-5°C (decomp.) was obtained.

The acetone soluble fraction was left in the cold for several days when a solid settled down which was filtered and washed with acetone (F-IV, 8.5 gm) mp. 275°C (decomp.). The filtrate was concentrated almost to dryness and the hygroscopic resinous material was macerated with ethyl
acetate and solvent washing were added to the benzene solution (Y) obtained earlier. The insoluble material was dissolved in alcohol, fractionally precipitated with ethyl acetate and ether. The precipitate thus obtained was warmed with alcohol cooled and filtered, when a brown powdered (FV-11.5 gm) mp. 270-73°C (decomp.) was obtained. The alcoholic filtrate and the alcohol ethyl acetate, ether solution were mixed and freed of the solvent to give a brownish water soluble semi solid (FVI - 60 gm).

The various fractions obtained above were chromatographed on paper chromatogram in different solvent system. The result are given in Table - I.

**TABLE - I**

Paper chromatography of crude saponin

(Spray reagent employed, Trichloroacetic acid (25%) in chloroform solution).

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Ethyl acetate: Butanol</th>
<th>Butanol</th>
<th>Butanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyridine:Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-I</td>
<td>0.09, 0.43</td>
<td>0.11, 0.52</td>
<td>0.74, 0.90</td>
</tr>
<tr>
<td>F-IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-V</td>
<td>0.43</td>
<td>0.52</td>
<td>0.90</td>
</tr>
<tr>
<td>F-VI</td>
<td>0.9 portion known a Bacoside B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-III</td>
<td>0.43 portioon known a Bacoside A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The supernatent (Z) obtained earlier was concentrated at 50°C in vacuo partitioned between butanol and water. The butanol solution was freed of solvent and the residue processed as given above. It finally gave an orange coloured powder (10.5 gm) mp. 230–33°C (decomp.) which showed only one spot Rf 0.43 on paper chromatography.

The procedure has thus led to the isolation of a saponin bacoside A (47.1 gm) correspond to spot Rf 0.43 and an other saponin bacoside B corresponding to spot Rf 0.09 and a mixture of these two.

**SEPARATION OF BACOSIDE A AND B**

The saponin mixture (1.5 gm) was chromatographed on silica gel (340 gm mixed with water 70% wt./wt.) using butanol-ethyl acetate-water (4:1:5) solvent system. Cuts of 10 ml were collected and later mixed together on the basis of paper chromatography result Table - II.

**TABLE - II**

Chromatography of crude saponin mixture

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>No. of spot</th>
<th>Rf</th>
<th>(wt. gm)</th>
<th>m.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16 - 19</td>
<td>1</td>
<td>0.43</td>
<td>0.525</td>
<td>250-1</td>
</tr>
<tr>
<td>20 - 24</td>
<td>2</td>
<td>0.43</td>
<td>0.487</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 - 31</td>
<td>1</td>
<td>0.09</td>
<td>0.450</td>
<td>203</td>
</tr>
</tbody>
</table>
**BACOSIDE A**

Bacoside A is obtained as colourless crystalline powder mp. 250 - 1°C (decomp.) from aqueous ethanol (60%). It is soluble in methanol and ethanol and very sparingly soluble in water. It forms a vigorous froth in aqueous suspension 

\[ [\alpha]_D = 42^\circ \text{ (80% ethanol)} \] (found C, 58.32%, H, 9.11%). \(C_{47}H_{76}O_{18}2H_2O\) requires C, 58.50%, H, 8.3%.

Bacoside A was refluxed with 65% methanol containing 8% sulphuric acid for four hours on water bath. After working the hydrolysate in the usual manner the aqueous portion was chromatographed on paper chromatogram (butanol-acetic acid-water) (4:1:5) when three spots of Rf 0.15, 0.20 and 0.24 for sugar were obtained along with aglycone fractions.

**ACID HYDROLYSIS OF BACOSIDE A**

Bacoside A (2 gm) was refluxed on water bath with 8% sulphuric acid in a methanol-water, 60:40 (100 ml) solution for four hours and extracted with chloroform. The aqueous portion was neutralised with barium carbonate chromatographed on paper in butanol-acetic acid-water (4:1:5) when three spots of Rf 0.15, 0.20 and 0.24 were obtained. The sugar corresponding to Rf 0.20 and 0.24 were identified as D-glucopyranose and L-arabinopyranose by optical rotation, osazone formation and by paper chromatography along with authentic sample. The third component Rf 0.15 was found to
be a disaccharide since on hydrolysis it gave glucose and arabinose which is examined as follows - the total neutral aqueous solution obtained above was concentrated filtered and the filtrate made upto 3.5 ml. This was chromatographed on a column and developed with water. Two ml cuts were collected and each fraction examined by paper chromatography as shown in Table III.

**TABLE - III**

Paper chromatography of sugar

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Spot</th>
<th>Rf</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>27 - 29</td>
<td>1</td>
<td>0.15</td>
<td>Unknown</td>
</tr>
<tr>
<td>30 - 31</td>
<td>2</td>
<td>0.15+0.20</td>
<td>Unknown+glucose</td>
</tr>
<tr>
<td>32 - 35</td>
<td>1</td>
<td>0.20</td>
<td>Glucose</td>
</tr>
<tr>
<td>36 - 38</td>
<td>2</td>
<td>0.20+0.24</td>
<td>Glucose+arabinose</td>
</tr>
<tr>
<td>39 - 42</td>
<td>1</td>
<td>0.24</td>
<td>Arabinose</td>
</tr>
</tbody>
</table>

**Fraction 39-42**: The solution on treatment with charcoal and concentrated gave a colourless residue $\alpha (D) + 107^\circ$ (water), phenyllosazone mp. 163$^\circ$.

**Fraction 32-35**: The total concentrate gave a phenyllosazone mp. 206 - 8$^\circ$C which did not depress the mp. of an authentic sample of glucosazone.
Fraction 27-29: (6 ml) were concentrated to 2 ml and hydrolysed with 5% sulphuric acid. The hydrolysate was neutralised and chromatographed on paper as usual. When the spot Rf 0.20 and 0.24 were obtained which correspond with spot of glucose and arabinose in the solvent system given earlier.

**Estimation of Total Sugar**

Bacosite A (30 mg) was hydrolysed with 8% sulphuric acid in aqueous ethanol (5 ml, 50%) for six hour and the mixture was extracted with chloroform solution in the usual way. The aglycone acidic solution was neutralised, concentrated and made up to 10 ml. An aliquot of the solution (1 ml) and 0.02N iodine (3 ml) were mixed and diluted to 6 ml with water, sodium carbonate (5%, 0.2 ml) in water bath at 21°C for 3 minute. After acidification with 0.5N sulphuric acid (1 ml), the residual iodine was titrated with 0.005N sodium thiosulphate. Thiosulphate solution consumed (2.3) ml = 1.06 mg of sugar which is equivalent to 35.3% of total sugar in bacosite A.

**Isolation of Aglycone of Bacosite A**

Bacosite A (10 gm) was hydrolysed with 8% sulphuric acid in a 60% methanolic solution (400 ml) methanol was distilled off and the residue extracted with chloroform. The
chloroform solution was washed, dried, and freed of solvent. The residue (6 gm) was macerated with a large excess of ether. Final evaporation of solvent gave an ether soluble fraction (2.8 gm) and ether insoluble fraction (3.2 gm). Thin layer chromatography of both the fractions were run. The ether soluble fraction showed five spots Rf 0.17 (violet), 0.28 (blue), 0.34 (blue), 0.40 (purple), 0.53 (violet). The ether insoluble fraction showed a brown streak (0.12 Rf.).

**ETHER SOLUBLE FRACTION**

The total fraction (2.8 gm) was chromatographed over alumina (85 gm) and a number of 75 ml fraction collected. Table IV.

**TABLE - IV**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Eluant</th>
<th>Wt. (mg)</th>
<th>Rf, TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Benzene:ether (1:1)</td>
<td>7</td>
<td>0.53</td>
</tr>
<tr>
<td>2.</td>
<td>Do</td>
<td>52</td>
<td>0.53, 0.40(F)</td>
</tr>
<tr>
<td>3.</td>
<td>Ether</td>
<td>130</td>
<td>0.53, 0.40</td>
</tr>
<tr>
<td>4.</td>
<td>Do</td>
<td>363</td>
<td>0.53, 0.40, 0.34(F)</td>
</tr>
<tr>
<td>5.</td>
<td>Ether:chloroform (1:1)</td>
<td>163</td>
<td>0.40, 0.34</td>
</tr>
<tr>
<td>6.</td>
<td>Do</td>
<td>122</td>
<td>0.34, 0.28</td>
</tr>
<tr>
<td>7.</td>
<td>Chloroform</td>
<td>94</td>
<td>0.28, 0.17</td>
</tr>
<tr>
<td>8.</td>
<td>Do</td>
<td>45</td>
<td>0.28, 0.17</td>
</tr>
</tbody>
</table>
9. Chloroform:ethyl acetate (1:1) 17 0.28, 0.17
10. Do 363 0.28, 0.17
11. Ethylacetate 361 0.17
12. Do 126 0.17
13. Ethyl acetate:methanol (4:1) 100 Streaking
14. Do 175 
15. Ethyl acetate:methanol (2:1) 25 
16. Do 92 

(F = faint)

Elutes residue of fraction 1-6 (0.83 gm) was mixed and chromatographed over silica gel (40 gm) using methanol (19:1) as eluting solvent. The fraction (8 ml each) collected are recorded in Table V.

**TABLE - V**

Chromatography of fraction 1-6 given in Table IV.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Fraction mixed</th>
<th>Wt. mg</th>
<th>Rf.TLC</th>
<th>Product isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1 - 8</td>
<td>Nil</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>9 - 13</td>
<td>45</td>
<td>0.53 with streak</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>14 - 22</td>
<td>140</td>
<td>'</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>23 - 25</td>
<td>16</td>
<td>'</td>
<td>-</td>
</tr>
</tbody>
</table>
5. 26 - 42 342 0.40, 0.32F with streak Bacogenin A4
6. 43 - 45 74 0.34, 0.28 (streak) -
7. 46 - 47 72 0.34, 0.28 -
8. 48 - 49 68 0.34, 0.28 Bacogenin A3
9. 50 - 51 49 0.28 with streak -

Fraction 7-12 (1.0 gm) from Table IV were also mixed and chromatographed over silica gel (60 gm) using benzene:methanol (9:1), sixty eight (10 ml each) were collected (Table VI).

**TABLE VI**

Chromatography of fraction 7-12 given in Table IV.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Fraction mixed</th>
<th>Wt. mg</th>
<th>Rf.TLC</th>
<th>Product isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1 - 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>7 - 30</td>
<td>62</td>
<td>0.34, 0.28(F)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>with streak</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>31 - 39</td>
<td>143</td>
<td>0.28 with streak</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>40 - 43</td>
<td>83</td>
<td>0.28, 0.19F</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>44 - 48</td>
<td>165</td>
<td>0.19</td>
<td>Bacogenin A2</td>
</tr>
<tr>
<td>6.</td>
<td>49 - 53</td>
<td>192</td>
<td>0.19, 0.15</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>54 - 60</td>
<td>285</td>
<td>0.15</td>
<td>Bacogenin A1</td>
</tr>
<tr>
<td>8.</td>
<td>61 - 68</td>
<td>78</td>
<td>0.15F with streak</td>
<td>-</td>
</tr>
</tbody>
</table>
ETHER INSOLUBLE FRACTION

It was obtained as a brown amorphous powder which was soluble in chloroform, acetone, ethyl acetate and methanol and insoluble in ether and benzene. It did not elute from alumina even with methanol:acetic acid 2:4 and about 80% was received from Florosil column (using chloroform:methanol) without any separation being effected. The purification through acetylation, lithium aluminium hydride reduction or diazomethane treatment did not give any encouraging result. Finally it was chromatographed over silica gel and elutes with ethyl acetate-methanol (9:1) but no crystallized product so far have been obtained.

Thus from bacaside A two sugars glucose and arabinose were isolated along with crystalline aglycone which is mixture of again four sapogenin named bacogenin A₁ mp. 240° C₃₀H₅₀O₄, bacogenin A₂ 222°, bacogenin A₃ mp. 190° C₃₀H₄₆O₃, and bacogenin A₄ 170°. The electron pattern of the bacogenin along with other data indicate that A₁ would be the most polar of the four aglycone and may thus be the true aglycone of bacaside A.

BACOSIDE B

Bacaside B was obtained as colourless powder from alcohol mp. 203°C (decomp.). It is sparingly soluble in ethanol, methanol and water and soluble in hot methanol. It
forths in aqueous suspension \( \alpha[D] + 8^0 \) 80% alcohol. Found 
C, 56.63%, H, 8.28% \( C_{47}H_{77}O_{19} \) \( 3H_2O \) requires C, 56.51%,
H = 8.21%. On hydrolysis give glucose and arabinose and aglycone containing bacoginin \( A_1, A_2, A_3 \) and \( A_4 \) and therefore has the same gross structure proposed for bacoside A. However bacoside B is dextro rotatory and bacoside A is laevorotatory and the homolytic activity of the former is twice that of the later.

**ACID HYDROLYSIS OF BACOSIDE B**

Bacoside B (5 gm) was hydrolysed with emulsion or dilute ethanolic sodium hydroxide solution. However, other condition similar to bacoside A, hydrolysis was effected with dilute methanolic sulphuric acid and neutral aqueous hydrolysate showed two spot \( R_f \) 0.20 and 0.24 on paper chromatogram in butanol:acetic acid:water (4:1:5) and developed with aniline hydrogen phthalate. The characterisation of these constituents units of the carbohydrate chain as glucose and arabinose was done by paper chromatographic separation comparison in various system. The total sugar in the molecule of bacoside B were estimated to be 35%. This was confirmed gravimetrically by the reduction of Fehling solution as cuprous oxide which gave a value of 34.7%. The remaining 65% of the molecule was accounted for the aglycone content.
ISOLATION OF AGLYCONE OF BACOSIDE B

The total aglycone fractions (3 gm) obtained by chloroform extraction of the acid hydrolysate of bacoside B was chromatographed over silica gel G and the result are given in Table VII.

**TABLE VII**

Chromatography of aglycone fractions of bacoside B.

<table>
<thead>
<tr>
<th>Eluants</th>
<th>Fraction No.</th>
<th>Vol. (ml)</th>
<th>Wt. gm.</th>
<th>TLC Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>1</td>
<td>75</td>
<td>0.56</td>
<td>Streaking</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>75</td>
<td>0.84</td>
<td>5 spot with streaking</td>
</tr>
<tr>
<td>Chloroform:</td>
<td>3</td>
<td>100</td>
<td>0.33</td>
<td>2 spots</td>
</tr>
<tr>
<td>methanol (10:1)</td>
<td>4</td>
<td>50</td>
<td>0.21</td>
<td>-do-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>50</td>
<td>0.25</td>
<td>-do- (faint)</td>
</tr>
<tr>
<td>Chloroform:</td>
<td>6</td>
<td>75</td>
<td>0.39</td>
<td>Brown streak</td>
</tr>
<tr>
<td>methanol (10:3)</td>
<td>7</td>
<td>75</td>
<td>0.067</td>
<td>-do-</td>
</tr>
</tbody>
</table>

Fraction 3, 4 and 5 (Table VII) were mixed together and the residue (0.79 gm) was rechromatographed over alumina in benzene. The residue from chloroform-ethyl acetate (1:1) elutes crystallized from chloroform as colourless needles mp. 222° C, Rf. 0.19 α[D] = -42° ethanol (found C, 75.76%; H, 10.60%; C₁₀H₁₆O₄ requires C, 75.95%; H, 10.54%, mixed mp. with bacogenin A₂ was undepressed. The residue from ethyl
acetate elute crystallized from benzene as colourless needles 
mp. 242° C, Rf 0.15 \([\alpha]_D = -70^\circ\) (chloroform) found C, 75.70\%  
H, 10.9\% \(\text{C}_{30}\text{H}_{50}\text{O}_4\) requires C, 75.95\%, H, 10.54\%, mixed mp. with bacogenin A\(_1\) was undepressed. Fraction 2 (Table VII) showing 
5 spots on TLC was fractioned over alumina and the major 
fraction was chromatographed over silica gel (40 gm) using 
benzene-methanol (9:1) (5 ml each) were collected and 
screened by TLC as shown in Table (VIII).

**TABLE - VIII**

Chromatography of fraction 2 given in Table VII.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Fraction mixed</th>
<th>Wt.mg.</th>
<th>Rf TLC</th>
<th>Product isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1 - 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>13 - 29</td>
<td>62</td>
<td>0.53F</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.28 with streak</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>30 - 38</td>
<td>125</td>
<td>0.40, 0.32F</td>
<td>Bacogenin A(_4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>with streak</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>39 - 45</td>
<td>74</td>
<td>0.34, 0.28</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>streak</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>46 - 50</td>
<td>68</td>
<td>0.34</td>
<td>Bacogenin A(_3)</td>
</tr>
</tbody>
</table>

Fraction 30-38 gave a residue which showed one spot 
Rf 0.40 identical with bacogenin A\(_4\) whereas fraction 46-50 
gave a single spot Rf 0.34 established the aglycone moiety 
identical to bacogenin A\(_3\).
It would thus seen clear that the bacoside A and bacoside B are identical in respect of their respective carbohydrate and the aglycone moieties and therefore bacoside B has the same gross structure and has been proposed for bacoside A.

The total benzene solution (Y) obtained in the fraction of original plant extract was concentrated, saponified with 10% alcoholic potash and extracted with ethyl acetate. The ethyl acetate solution on concentration formed a thick gel which was cooled and filtered. The filtrate gave a further quantity of the gelatinous precipitate on concentration. The total precipitate was again dissolved in hot ethyl acetate cooled and filtered. A crystalline powder mp. 302 - 4°C (decomp.) thus obtained as a fine colourless needle compound D from hot alcohol yield 5.8 gm, mp. 315°C (found C, 79.3% H, 10.7% calculated for C₃₀H₄₈O₃, C, 78.9% H, 10.6%.

The total mother liquor obtained above was concentrated to dryness and the residue (31 gm) was chromatographed on alumina (500 gm) elution was carried out as shown in Table IX.
# TABLE IX

Chromatography of unsaponified matter from benzene solution (31 gm).

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluants</th>
<th>Wt. (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 3</td>
<td>Petroleum ether</td>
<td>5.48</td>
</tr>
<tr>
<td>4 - 5</td>
<td>Benzene:Petroleum ether (1:1)</td>
<td>1.38</td>
</tr>
<tr>
<td>6 - 7</td>
<td>Benzene:Petroleum ether (2:1)</td>
<td>4.49</td>
</tr>
<tr>
<td>8 - 9</td>
<td>Benzene:Petroleum ether (4:1)</td>
<td>2.44</td>
</tr>
<tr>
<td>10</td>
<td>Benzene</td>
<td>1.17</td>
</tr>
<tr>
<td>11</td>
<td>-do-</td>
<td>0.39</td>
</tr>
<tr>
<td>12 - 13</td>
<td>Benzene:Chloroform (1:1)</td>
<td>1.33</td>
</tr>
<tr>
<td>14 - 15</td>
<td>Benzene:Chloroform (1:2)</td>
<td>1.22</td>
</tr>
<tr>
<td>16 - 18</td>
<td>Benzene:Chloroform (1:4)</td>
<td>1.5</td>
</tr>
<tr>
<td>19</td>
<td>Chloroform</td>
<td>5.66</td>
</tr>
<tr>
<td>20 - 21</td>
<td>Chloroform:Ethyl acetate (1:1)</td>
<td>0.39</td>
</tr>
<tr>
<td>22 - 23</td>
<td>Ethyl acetate</td>
<td>0.47</td>
</tr>
<tr>
<td>24 - 25</td>
<td>Ethyl acetate: Methanol (4:1)</td>
<td>0.61</td>
</tr>
<tr>
<td>26 - 27</td>
<td>Ethyl acetate: Methanol (2:1)</td>
<td>1.08</td>
</tr>
</tbody>
</table>

Oily

Crystal with matrix

Resinous

Powder
The 1–10 elutes were oily and were not worked further. The residue from 11–19 crystallized from methanol and melted in the range of 120–32°C. These were mixed (738 mg) and rechromatographed over alumina using benzene:chloroform (1:2) as eluants. Colourless needles were obtained yielding 350 mg. This fraction proved to be mixed crystal of β-sitosterol and stigmasterol and were resolved by the procedure given below.

The mixture crystallized from alcohol yield 1.75 gm, mp. 146–50°C. The crystalline methanol was acetylated with acetic anhydride and pyridine in the usual manner. The crude acetate was dissolved in ether and treated with bromine in glacial acetic acid for 12 hours at room temperature. The crystalline insoluble bromide thus obtained filtered and washed with ether. It was debrominated by refluxing with zinc and acetic acid for one hour then deacetylated with 5% alcoholic potash and crystallization from alcohol. Colourless needles were obtained yield 780 mg as compound E mp 170°C.

The ether filtrate from the above was evaporated to dryness. The residue was debrominated and deacetylated. The resultant product (870 mg) was purified by chromatography on alumina. The benzene chloroform (1:2) elute gave colourless needles from alcohol yield 850 mg compound F mp. 137°C.

The solid material from fraction 26 and 27 was rubbed with little warm acetone cooled and filtered. The
powder crystalline from alcohol as colourless needles (930 mg) mp. 315°C. It was found to be identical with compound D obtained earlier.

Along with these isolation a new hydrolysable tanin has been searched out for the first time from this plant. It is well known that naturally occurring hydrolysable tanin usually contain depside links which get alcoholysed during extraction with alcohol. The technique applied for its extraction is as follows.

The residue left after extraction of material with chloroform was extracted with acetone in soxhlet apparatus. The combined acetone extract (400 ml) was concentrated under reduced pressure (100 ml) and ether was added. Some impurities which settled down first were removed and to the decanted solution more ether was added when brown precipitate was obtained. This on repeated crystallization from acetone and ether mixture gave an almost creemish microcrystalline, chromatographically solid homogeneous which on TLC gave Rf 0.32 (solvent benzene:methanol 1:1) sprayed with iodine, mp. 235°C marked as compound G.
B. CHARACTERISATION OF THE CONSTITUENTS

CHARACTERISATION OF COMPOUND A (Bacoside A)

The dried and powdered plant of *Bacopa monnieri* (6 Kg) after extraction with benzene was exhaustively extracted with 95% ethanol (3 lt.), the combined alcoholic extracts were concentrated under reduced pressure. The concentrated brown coloured thick mass was boiled with water to make it dilute and filtered. The filtrate was then treated with basic lead acetate solution. The heavy golden yellow precipitate of lead tannate, gallate etc. was filtered and the basic lead acetate was removed by saturating the solution with hydrogen sulphide gas and filtered off the lead sulphide. The filtrate was boiled and simultaneously all the traces of H₂S gas were removed by passing a current of air through it. The concentrated aqueous solution was then concentrated to one third of its volume and successively extracted with light petroleum ether (bp 40-60°C) to get rid of the resinous and waxy material. The clear solution was concentrated to vacuum yielding (6 gm) bacoside A. It is colourless crystalline powder mp. 250-1°C (decomp.). It is soluble in alcohol and is insoluble in ether acetone etc. It forms a vigorous forth in aqueous suspension [\(\alpha\)]_D - 42° (80% ethanol). Found C 58.32%, H 9.11%, C_{47}H_{76}O_{18} requires C 58.50%, H 8.3%.

The following colour reaction have been exhibited
by the bacoside A:

(a) **Libermann Reaction**

A speck of bacoside A was dissolved in cold acetic anhydride and treated with a drop of concentrated sulphuric acid. A display of colour followed by a green fluorescence was observed.

(b) **Tortelli-Jaffe Reaction**

On pouring a layer of 2% solution of bromine in chloroform to a solution of bacoside A in acetic acid a brown colouration appeared at the interface.

(c) **Tollen's Reaction**

About 10 mg of bacoside A was dissolved in 1 cc of pure pyridine and diluted with an equal amount of water. The solution was treated with .5 cc of tollen's reagent and then allowed to stand at room temperature. The reaction occurred gradually and definitely deposition of silver was noticed with in 30 minute.

(d) **Beljet Reaction**

3 ml of an alcoholic solution of picric acid were mixed with a solution of bacoside A in alcohol. To this a few drops of sodium hydroxide were added and the whole
mixture was warmed with occasional shaking. A red orange colour was obtained.

(e) Legal Test 20

A solution of bacoside A in distilled water was treated with freshly prepared sodium nitroprusside solution followed by the addition of a drop or two of sodium hydroxide solution. A deep ruby red colour developed.

(f) Raymond Reaction 21

A pinch of bacoside A was dissolved in 1 ml. of 50% alcohol. To this solution were added two drops of a 1% solution of m-dinitro benzene in absolute alcohol and cooled in ice bath; After ten minute a drop of 20% sodium hydroxide solution was added when a bluish violet colour was formed.

HYDROLYSIS OF BACOSIDE A

Bacoside A (2 gm) was hydrolysed by refluxing with 500 ml of 50% aqueous methyl alcohol containing 5% sulphuric acid, for a period of 4 hours. The glycoside did not dissolve completely at first but after heating for a short while the solution become clear and homogeneous with a pale yellow colour deepened on further heating. Completion of hydrolysis was ascertained by examining sugar content after every three hours in a 1 cc sample from the hydrolysing mixture with
Fehling solution and weighing the reduced cuprous oxide each time till become constant. Methyl alcohol was then removed by distillation under reduced pressure and the hydrolysed mixture was thoroughly extracted with ether. The ethereal layer was washed with water free of mineral acid, dried over anhydrous sodium sulphate and the solvent distilled off yielding a semi solid substance (2.5 gm).

**TREATMENT OF AQUEOUS PHASE**

After extracting the aglycone from the hydrolysed mixture with ether, the aqueous phase was treated with pure barium carbonate till a pH at 6.0±0.5 was maintained and the precipitated barium sulphate filtered. The filtrate was concentrated in vacuum to a thick syrup. The later was again refluxed with dilute sulphuric acid for eight hours. The sulphuric acid was then removed with barium carbonate as before and the heavy barium sulphate precipitate filtered off. The resulting filtrate was concentrated under reduced pressure to a semi solid mass (7.2 gm) and dried in a vacuum desiccator over P₂O₅. In order to get rid of the inorganic salts the dry residue was extracted with 500 ml of redistilled dry pyridine at 100°C for 30 minute. The extract was cooled and filtered. The pyridine solvent was completely removed under diminishing pressure at a temperature not exceeding 45°C yielding (4.5 gm) of solid mixture of sugars.
COLOUR TESTS OF SUGARS

1. Molisch Test

A little sugar mixture was dissolved in 1 cc. of water in a test tube and 2 drops of 10% solution of α-naphthol in alcohol were mixed with it. About 1 cc. of concentrated sulphuric acid was carefully trickled down the side of the test tube forming a layer beneath the aqueous solution without causing disturbance. After a couple of minutes a red violet ring appeared at junction of two layers.

2. Fehlings Solution

On adding 2 ml of Fehling solution (prepared by mixing equal volumes of copper sulphate solution and alkaline sodium potassium tartarate solution) to a solution of 0.1 gm of the sugar mixture and gently boiling for two minutes a red precipitate of cuprous oxide was formed.

3. Barfoed's Reagent

A test tube containing Barfoed's reagent and solution of the sugar mixture, 1 ml each, was heated in a beaker of boiling water. A red precipitate of cuprous oxide was formed within two minutes. (Barfoed's reagent was prepared by dissolving 13.3 gm of crystalline neutral copper acetate in 200 ml of one per cent acetic acid solution).
4. **Tollens Test**

To an aqueous solution of sugar mixture in a clean test tube, 5 ml of freshly prepared ammonical silver nitrate solution were added. The mixture was warmed on a water bath for a few minutes. A black precipitate of metallic silver was formed.

**Test of Glucose**

To 2 cc. of the aqueous solution in a test tube, solid lead acetate was added and heated to boiling. It was cooled and on addition of 5 ml of dilute NH₄OH, a rose pink colour developed, indicating the presence of glucose.

**PREPARATION OF OSAZONE**

Sugar mixture (0.2 gm), pure phenyl hydrazine hydrochloride (0.4 gm) and sodium acetate (0.7 gm) were placed in a test tube with 4 ml of distilled water and allowed to stand in boiling water for 10 minute periodically shaking, when a bulky yellow precipitate of osazone separated. This on crystallisation from alcohol melted at 203°C which was not lowered by admixing with an authentic specimen of glucosazone.

**OXIDATION WITH NITRIC ACID**

One gram of the sugar mixture was treated with
nitric acid (3 ml) and set aside in boiling water until red fumes were evolved. It was heated at 70°C for another 25 minutes diluted with water and the white precipitate filtered and crystallized from 50% hot alcohol mp. 209°C.

**ANALYSIS OF THE SUGAR MIXTURE**: (Paper Chromatography)

Whatman no. 1 filter paper was used for preparing strip chromatograms. The paper was cut into pieces of 50 cm. length and 30 cm. width. Approximately 1% (v/v) solution of the sugar mixture as well as of the glucose, galactose, arabinose as standard sugars were prepared with a fine glass capillary. These solutions were introduced in the form of circular spot, 4 cm. apart on a horizontal line drawn, 5 cm. from the top edge of the paper strip. In this manner, the strip chromatogram was prepared and vertically hung down from the side of the trough containing a mixture of n-butanol: acetic acid:water (4:1:5). Both the strip and the trough were placed inside a chamber which was fully saturated previously with the vapour of the above solvent mixture and the chromogram was irrigated for eight hours. Thereafter, it was taken out of the chamber and the exact position of the solvent boundary was marked. The solvent was dried off in an oven at 105°C. In order to reveal the new positions of the component sugars of the solutions, the paper strip was sprayed rapidly and evenly with aniline hydrogen phthalate and replaced in the
oven (105°C) for ten minutes. Different sugars appeared as dark brown spots on the white ground. The strip chromatogram was then washed first with distilled water and afterwards in running tap water for about three fourth of an hour and finally dried. By matching the location of the spot position of various constituents of the sugar mixture on the chromatogram with those of the standard sugars the presence of glucose and arabinose in the mixture was confirmed. Then Rf values were calculated as 0.20 and 0.24 respectively.

ESTIMATION OF TOTAL SUGAR

Bacoside A (30 mg) was hydrolysed with 8% sulphuric acid in aqueous methanol (50%, 50 ml) for 6 hours and the mixture was extracted with chloroform solution in the usual way the aglycone residue (18.6 gm) about (62%) was obtained. The aqueous acidic solution was neutralised, concentrated and made up to 100 ml. An aliquot of solution and 0.02N Iodine (3 ml) were mixed and diluted with water. Sodium carbonate (5%, 2 ml) was then added slowly and the flask was placed in water bath at 25°C for 3 minutes. After acidification with 0.5N sulphuric acid (1 ml) the residual iodine was titrated with 0.005N sodium thiosulphate. This sulphate solution consumed (2.3 ml) = 1.06 mg of sugar which is equivalent 35.3% of total sugar in bacoside A.
Purification of the aglycone fractions (Bacogenins)

Bacoside A (10 gm) was hydrolysed with 8% sulphuric acid in a 60% methanolic solution. Methanol was distilled off and the residue extracted with chloroform. The chloroform solution was washed dried and freed of solvent. The residue (6 gm) was macerated with a large excess of ether. Finally evaporation of solvent gave an ether soluble fraction (2.8 gm) and an ether insoluble fraction (3.2 gm). The ether soluble fraction was analysed through thin layer chromatography over alumina and different number of fractions were collected. Elute residue from (1-6) (0.83 gm) was mixed and chromatography over silica gel (40 gm) using methanol as eluting material. The fraction from 48-49 elute were mixed and on thin layer chromatography showed a spot Rf (0.34) mp. 190°C designated as bacogenin A3.

Fraction 7-12 (1 gm) were also mixed and chromatographed over silica gel (60 gm) using benzene:methanol (9:1). Fraction of (44-48) elutes were mixed and on TLC showed one spot Rf 0.19 mp. 222°C designated as bacogenin A1. The (54-60) fractions were also mixed and on TLC showed a spot Rf 0.15 mp. 242°C known as bacogenin A2.

Thus bacoside A on hydrolysis gave a mixture of aglycones (sapogenins) named bacogenin A1 mp. 240°C, bacogenin A2 mp. 220°C, bacogenin A3 190°C, and bacogenin A4 mp. 175°C.
The elution pattern of bacogenin along with other data indicates that A₁ would be the most polar of the four aglycone and may thus the true aglycone of bacoside A.

CHARACTERISATION OF BACOGENIN A₁

It is sparingly soluble in benzene and ether and soluble in all other aqueous solvent. Crystallized from benzene mp. 242° C. It gave a violet colour with thionyl chloride (Noller's reagent) and a yellow colour with tetra nitromethane showing unsaturation of molecule \([\alpha]_D - 70\) (chloroform)

\(\nu_{\text{max}}\) KBr, 3410, 1730, 1660, 1450, 1375 (d) 1080, 820 cm\(^{-1}\)

NMR peaks 45.5, 52, 58, 76, 83, 91, 96, 192, 223, 235, 242, 254 (quartet) and 320 cycle/sec. PMR (ppm) .783, .875, 1.06, 1.26, 1.38, 1.53 (3H each S 7xMe), 1.63 (3H, d, J 1.5 H₂C = C-Me), 2.2 (3H, bs CH₂-CHOCH), 3.18 (1H, qJ, 9.5 and 5Hz-CHO), 3.95 (2H, ABq JAB 11 Hz CH₂-O-), 5.35 (1H qJ 1.5 Hz H₂C=C-H),

MS m/e, 472 (M\(^+\)), 457 (m-15), 439 (M-15-18), 207, 189, 125, base peaks, 110, 107 (125-18 m at 91.4), Found C-75.74%, H-10.8%, \(C_{30}H_{48}O_4\) requires C-76.27%, H, 10.16%.

DERIVATIVES OF BACOGENIN A₁

1. Di-o-acetyl Bacogenin A₁

Bacogenin A₁ on reaction with acetic anhydride and pyridine at room temperature for 24 hour gave colourless
needles from ethanol mp. 220°C \( \nu_{\text{max}} \) 3440, 1730, 1460, 1440, 1375, 1365, 1240, 827 cm\(^{-1}\) (CCl\(_4\)). NMR peaks, 51.5(2), 53.5, 75.5, 82, 92, 96, 252, 243, 255, 268, 280 (quartet) and 320 (double) cycles/sec. Found C-72.5%, H-9.67%, acetyl 15.6%, \( \text{C}_{34}\text{H}_{54}\text{O}_{6} \) requires C-73.11%, H-9.67%.

2. Dihydro Bacogenin A1

Bacogenin A1 (11.2 mg) on hydrogenation with platinum in acetic acid colourless needles mp. 192°C were obtained from aqueous ethanol. Found C-75.26%, H-10.72%, \( \text{C}_{30}\text{H}_{52}\text{O}_{4} \) requires C-75.63%, H-10.92%.

3. 2,4-Dinitrophenyl Hydrazone of Bacogenin A1

Bacogenin A1 (25 mg) was left overnight with DNP reagent (1 ml of solution of 2,4-dinitrophenyl hydrazine) (.25 gm) in methanol (5 ml) containing 0.2 ml concentrated sulphuric acid. The orange red crystals were filtered washed with methanol and chromatographed over alumina. The benzene elute gave red needles (10 mg) from ethanol mp. 110-12°C.

4. Lithium Aluminium Hydride Reduction of Bacogenin A1

A solutionbacogenin (100 mg) in ether (10 ml) was added with stirring to a suspension of lithium aluminium hydride (200 mg) in ether (15 ml) and refluxed for 4 hours.
The excess of lithium aluminium hydride was decomposed with ethyl acetate reaction mixture acidified extracted with the same solvent and worked up as usual. The residue crystallized from ethyl acetate ether as colourless needles mp. 252°C.

\[ \delta_{\text{max}} \text{(Najol)} 3400, 3280, 1460, 1370 \text{ and } 817 \text{ cm}^{-1} \]. Found C-74.91%, H-10.52%, C\(_{30}\)H\(_{52}\)O\(_4\) requires C75.63%, H-10.92%.

5. **RuO\(_4\)-NaIO\(_4\) Oxidation of Di-o-acetyl Bacogenin A\(_1\)**

A mixture of di-o-acetyl bacogenin A\(_1\) (280 mg) in acetone (15 ml) and RuO\(_4\) (~50 mg) in carbon tetrachloride (15 ml) was stirred with an aqueous of NaIO\(_4\) (500 mg, 8 ml) for five hours. The aqueous layer was separated and the excess reagent decomposed by the addition of isopropanol (2 ml). The precipitated RuO\(_4\) was filtered and the filtrate washed with water dried and evaporated. The residue (310 mg) shows two major components designated as UA\(_1\) and LA\(_1\) (Rf = .34, and .1 in C\(_6\)H\(_6\) + 5% Methyl alcohol) and was chromatographed over silica gel. The benzene:chloroform (1:1) elutes furnished UA\(_1\) (80 mg) while chloroform-ethylacetate (3:1) elutes yielded LA\(_1\) (85 mg) both as colourless powder.

**Product UA\(_1\)**

\[(\alpha)_{D} = -17^\circ (C, 1\% \text{ ethyl alcohol}): \delta_{\text{max}}(\text{KBr}) 3500, 1762, 1730, 1250, 1035 \text{ cm}^{-1}, \text{PMR (ppm)} .85, 1.31 (6\text{H each, S, 4xMe}), .91, 1.05, 1.25, 1.46, (3\text{H each, S, 4xMe}), 2.05, 2.06\]
(3H each, S, 2x-0 COCH₃), 2.64 (1H, d, J = 12 Hz - CO-CH),
4.45 (2H, AB quartet J = 12 Hz, -CH₂-OAc), 4.52 (1H, m-CHOAc):
MS m/e 588 (M⁺) 545, 528, 497, 502, 485, 475, 459, 442, 433,
415, 399, 373, 355, 339, 313, 279, 203, 189, 178, 161, 135,
121, Found C-69.25%, H-9.15%, C₃₄H₅₂O₈ requires C-69.38%,
H-8.91%.

Product LA₁

υ max(KBr), 3450, 2350, 1750, 1250 cm⁻¹: MS m/e (M⁺)
not visible 517, (M⁺ 87) 500, 475, 433, 415, 373, 355, 337,
313, 149, PMR (ppm) .88 (6H, S, 2x-CH₃), .94, 1.14, 1.29,
1.48, 1.65 (3H each S, 5x-CH₃), 2.00, 2.066 (3H each at
S, 2x-0 COCH₃), 2.25 (3H, S, -COCH₃), 4.5 (2H, AB quartet
J = 12 Hz -CH₂OAc), 4.4 (1H, m-CHOAc). Found C-47.22%,
H-8.90% C₃₄H₅₂O₉ requires C-67.54%, H-8.67%.

CHARACTERIZATION OF BACOCENIN A₂

Bacogenin A₂ is an isomer of bacogenin A₁ differing
in the configuration at C-20. It also gave positive (Noller's
reaction) showing triterpinoids class of compound. It is
soluble in all organic solvent and sparingly soluble in
benzene and ether, mp. 220°C [α]D = -44⁰ (C, 1% ethyl alcohol)
υ max(KBr), 3350, 2925, 2850, 1750, 1465, 1375, 1200, 1040,
970, 844, 824, 785, 752, PMR (ppm) .80, .90, .983, 1.25, 1.31
(3H each S, 5x-CH₃), 1.15 (6H, S 2x-CH₃), 1.73 (3H, d J 1.5 Hz - C=CH₃), 2.2 (3H, bs-CH-CO-CH₂), 3.23 (1H q J 10, 5 Hz -CH-O-), 3.966 (2H, AB q J 11Hz, -CH₂-o-), 5.30 (1H, q J, 1.5 Hz -C=CH₂), MS: m/e 472 (M⁺) 457, 439, 209, 189, 180, 125 (base peaks) 110, 107 Found C-75.98%, H-10.35%. C₃⁰H₄₈O₄ requires C-76.27%, H-10.16%.

DERIVATIVES OF BACOGENIN A₂

Di-o-acetyl bacogenin A₂

Bacogenin A₂ on reaction with pyridine and acetic anhydride at room temperature for 24 hours gave needles from ethanol mp. 210°C.

RuO₄-NaIO₄ oxidation of di-o-acetyl bacogenin A₂

A solution of di-o-acetyl A₂ (250 mg) in acetone (12 ml) was stirred with a solution of RuO₄ (80 mg) in carbon tetrachloride (12 ml) and a solution of NaIO₄ (50 mg) in water (8 ml) for 5 hours. The reaction product showed two major product (TLC in benzene + 5% methanol) designated as UA₂ and LA₂ in discarding order of Rf value. On chromatography on silica gel product UA₂ was obtained chloroform elutes as colourless powder and product LA₂ was eluted in chloroform: ethyl acetate (3:1) fraction and crystallized from hexane-benzene as colourless rhombic mp. 198-200°C.
Product UA2

\((\alpha)_D = +84.6^\circ\) \(\text{C, 1\% ethyl alcohol}\) \(\lambda_{\text{max}}\) (KBr) 3500, 1760, 1740, 1250, 1035, 985 cm\(^{-1}\), PMR (ppm) .89, 1.3, (6H each, S, 4x-CH\(_3\)), .94, 1.10, 1.40, 1.45 (3H each, S, 4x-CH\(_3\)) 2.0, 2.15 (3H each, S, 2x-OCOCH\(_3\)), 4.55 (1H, m, -CHOAc), 4.57 (2H, AB quartet, J=12 Hz -CH\(_2\)OAc). MS: m/e 588, 528, 512, 502, 488, 475, 459, 442, 433, 415, 399, 373, 355, 337, 313, 279, 203, 189, 161, 135, 121, 114 (Found C=68.90\%, H=9.22\%, C\(_{34}\)H\(_{52}\)O\(_8\) requires C=69.38\%, H=8.91\%).

Product UL2

mp. - 198-200°C \(\lambda_{\text{max}}\) (KBr) 3275, 2450, 1740, 1250, 1040, PMR (ppm) .89 (9H, S, 3x-CH\(_3\)), 1.09, 1.366, 1.43, 1.55 (3H each, S, 4x-CH\(_3\)), 2.09, 2.01 (3H each, S, 2x-OCOCH\(_3\)), 2.3 (3H, S, -COCH\(_3\)), 4.50 (1H, m-CHOAc), 4.52 (2H, AB quartet J=13 Hz -CH\(_2\)OAc). MS: m/e M\(^+\) (not visible), 517 (M\(^+\)-87) 500, 475, 433, 415, 373, 355, 337, 313, 135, 78, Found C=67.86\%, H=8.87\%, C\(_{34}\)H\(_{52}\)O\(_9\) requires C=67.54\%, H=8.678\%.

CHARACTERISATION OF BACOGENIN A3

Melting point 190°C \(\lambda_{\text{max}}\) (KBr) 3344, 2907, 2825, 1630, 1450, 1360, 1276, 1221, 1027, 1000, 940, 905, 835 cm\(^{-1}\) \(\lambda_{\text{max}}\) 230 nm (E9630), NMR (ppm) .78, .84, .98, 1.03 (H each, S, 4x-C-Me), 1.53 (6H, d, J, 1.5 Hz, 2G=C-Me), 1.76 (3H, d, J,
1.5 Hz -C=CH₂), 3.21 (1H, m -CH₂O-), 4.1 (4H broad, S, 2-CH₂O), 5.43 (1H, m -C=C-H), MS: m/e 454 (M⁺) 439 (M-15) 436, (M-18) 411, 334, 318, 316, 301, 274, 257, 241, 207, 189, 175, 173, 161, 149, 135, 121. Found C-79.73%, H-10.45%, C₃₀H₄₆O₃ requires C-79.27%, H-10.20%.

ACETYL BACOGENIN A₃ (lb)

A mixture of bacogenin A₃ (250 mg), pyridine (2.5 ml) and acetic anhydride (2.5 ml) was allowed to stand over night. After usual work up, the product was crystallized from methanol, mp. 220°C λ_{max} EtOH 236 nm.

NMR (ppm): 0.86 (9H, S, 3C-CH₃), 1.05 (3H, S, C-CH₃), 1.53 (6H, d, J 1.5 Hz, 2C=C-CH₃), 1.78 (3H, d, J 1.5 Hz C=C-CH₃), 2.05 (3H, S, -OCOCH₃), 4.1 (4H, broad, S, 2-CH₂O), 4.48 (1H, m, -CHOAc) 5.45 (1H, m, -C=C-H).

MS: m/e (M⁺ absent) 316, 301, 273, 257, 241, 203, 189, 175, 173, 161, 149, 147, 135, 121.

OZONOLYSIS OF ACETYL BACOGENIN A₃

A solution of acetyl bacogenin A₃ (lb) (100 mg) in dichloromethane (10 ml) containing pyridine (.02 ml) was saturated with ozonised oxygen at -80°C for 45 minute. The solvent was evaporated off and the product crystallized from
methanol as colourless needles mp. 246°C \( \lambda_{\text{max}}^{(\text{KBr})} \) 1730, 1670, 1635, 1265, 775 cm\(^{-1}\), \( \lambda_{\text{EtOH}} \) (max): 243 nm (E 1150). NMR (ppm) 0.86 (9H, S, 3-C-CH\(_3\)), 1.05 (3H, S, C-CH\(_3\)), 2.05 (3H, S, -OOCCH\(_3\)), 2.15 (3H broad, S, C=C-CH\(_3\)), 4.15 (2H, S, -CH\(_2\)O-), 4.5 (2H, AB q J 14 Hz -CH\(_2\)O and 1H, m, -CHOAc), 9.96 (1H, S, -CHO), MS: m/e 470 (M\(^+\)) 189, 175, 161, 149, 135, 121, 107, 93, 81, 69.

**HYDROGENATION OF ACETYL BACOGENIN A\(_3\)**

Acetyl bacogenin A\(_3\)(1b) (125 mg) was hydrogenated over Adam's catalyst in chloroform-ethyl acetate (1:1, 10 ml) for four hours. The product was purified by paper liquid chromatography on silica gel with benzene-methanol (9:1) and crystallized from benzene:petrol as colourless needles mp. 145°C (100 mg) \( \lambda_{\text{max}}^{(\text{KBr})} \) 3350, 1725, 1250 cm\(^{-1}\). NMR (ppm) 0.85 (9H, S, 3-C-CH\(_3\)), 1.08 (3H, S, C-CH\(_3\)), 0.85 (12H, d, J 7 Hz, 4-CH\(_2\)CH\(_3\)), 2.03 (3H, S, -OOCCH\(_3\)), 2.18 (2H, AB q J 16 Hz -CH\(_2\)CO-) 3.96 (2H, AB q J 12 Hz -CHOAc), 4.48 (1H q J, S and 9 Hz -CHOAc). MS: m/e 502 (M\(^+\)), 472, 443, 428, 412, 399, 390 (M side chain + H), 372 (390-H\(_2\)O), 357, 339, 333, 330, 315, 312, 297, 262, 205, 203, 189, 135, 121, 109, 107, 95, 93.

The product (4a)(50 mg) was acetylated with acetic anhydride-pyridine to furnish a diacetate (4b)(30 mg): \( \lambda_{\text{max}}^{(\text{KBr})} \) 1727, 1244 cm\(^{-1}\). NMR (ppm) 1.96, 2.03 (3H each, S, 2-OOCCH\(_3\)), 4.43 (2H, AB q J 12 Hz -CH\(_2\)OAc), 4.55 (1H, m -CHOAc). The diacetate
(4b) was saponified with 2M ethanolic KOH at room temperature for 16 hours. The deacetylated product (4c) (45 mg) was obtained as a colourless powder.

\[(\text{KBr})_\text{max} 3344, 1727 \text{ cm}^{-1}\] NMR (ppm) \(80, .90, 1.00, 1.10\) (3H each S, 4-C-CH\(_3\)), 0.85 (12H, d J, 7 Hz 4-CH-CH\(_3\)), 2.18 (2H, AB q J 16 Hz -CH\(_2\)CO-), 3.24 (1H, m-CHOH), 4.0 (2H, AB q J 12 Hz -CH\(_2\)OH), 411, 375, 348, (M-side chain + H) 330, 315, 297, 284, 279, 256, 213, 207, 189, 185, 149, 141, 113 (side chain) 99, 97, 85. Found C-78.32%, H-11.60% C\(_{30}\)H\(_{52}\)O\(_3\) requires C-78.20%, H-11.37%.

**CHARACTERISATION OF BACOGENIN A\(_4\)**

Bacogenin A\(_4\) mp. 175°C C\(_{30}\)H\(_{46}\)O\(_3\) (M\(^+\) 454) was found to be the major compound in aglycone mixture. It showed \(\lambda_\text{max} 269, 279, 291 \text{ nm}\) and formed a monoacetate mp. 212°C C\(_{32}\)H\(_{48}\)O\(_4\). It has been identified as ebeline lactone from its chemical and physiological data and finally by the direct comparison (TLC, mixed mp.; PMR and IR) of mono-o-acetyl bacogenin A\(_4\) with mono-o-acetyl ebeline lactone which was obtained from Zizyphoside.

**CHARACTERISATION OF COMPOUND B (Bacoside B)**

It is obtained as colourless needles from alcohol mp. 203°C (decomp.). \([\alpha]_D = +8^\circ\) (80% alcohol). It is soluble in hot methanol and insoluble in alcohol and water. Found
The following colour reaction have been shown by the glucoside bacoside B.

1. **Liebermann Reaction**

   A small amount of bacoside B was dissolved in cold acetic anhydride and treated with a drop of concentrated sulphuric acid. A display of colour followed by a green fluorescence was observed.

2. **Tollen's Reaction**

   About (8 mg) of bacoside B was dissolved in 1 cc of pure pyridine and diluted with an equal amount of water. The solution was treated with the .5 cc of Tollen's reagent and then allowed to stand at room temperature. The reaction occurred gradually and definitely deposition of silver on the wall of vessel was noticed within half an hour.

3. **Legal Test**

   A solution of bacoside B in distilled water was treated with freshly prepared sodium nitroprusside solution followed by the addition of a drop or two of sodium nitroprusside solution. A deep ruby red colour developed.

**HYDROLYSIS OF BACOSIDE B**

It was hydrolysed with 65% methanol containing 8%
sulphuric acid. The aqueous portion of the hydrolysate was chromatographed on paper chromatogram (butanol:acetic acid: water 4:1:5) gave two spots Rf 0.20 and 0.24 developed with aniline hydrogen pthalate which identified as glucose and arabinose with spot of authentic sample. The sugar were the same as isolated from bacoside A.

The aglycone mixture obtained after the hydrolysis of bacoside B was found to contain four same sapogenin as in case of bacoside A i.e., bacogenin A₁, bacogenin A₂ and bacogenin A₃ etc. The technique for their isolation and characterisation of component is almost same to that of the bacoside A which is well elaborated previously.

CHARACTERISATION OF COMPOUND C : (D-mannitol)

It is obtained from alcohol extract of plant on concentration under reduced pressure at 50°C as crystalline powder. It is recrystallized from dilute methanol as colourless needles mp. 166°C. It was soluble in water and insoluble in rest of the solvent Found C-39.7%, H-7.3%, C₆H₁₁O₆ requires C-39.56%, H-7.69%. With ferric chloride solution it gave yellow colour which shows that it is a poly phenolic compound.

DERIVATIVES

ACETATE

10 mg of substance was boiled with acetic anhydride
(2 ml) and freshly fused sodium acetic acetate (25 mg) for 4 hours. The reaction product after cooling was diluted with water and then filtered. The clear solution was made ammonical by addition of dilute ammonia when the acetylated compound was precipitated. It was extracted with ether and the ethanol solution dried over anhydrous sodium sulphate. Ether was distilled off to get crude residue which upon repeated crystallization from alcohol separated as colourless needles mp. 124°C. It did not depressed the melting point on admixture with authentic specimen.

CHARACTERISATION OF COMPOUND D : (Betulinic acid)

It is obtained as crystalline silky needles mp. 31.5°C. It was soluble in hot ethyl acetate, alcohol and methanol, fairly so in acetone and chloroform and insoluble in benzene. Found C-78.69%, H-10.55%. C_{30}H_{48}O_{3} requires C-78.9%, H-10.94% \( [\alpha]_D = +7^\circ \) (in pyridine).

COLOUR REACTIONS

1. Liebermann Burchard Reaction\(^7\) : A small amount of solution in chloroform developed deep red colour when concentrated sulphuric acid and few drops of acetic anhydride.

2. With thionyl chloride it gave a red colour which darkened and developed a violet tinge in 3-4 minute.
PREPARATION OF DERIVATIVES

1. Acetate

10 mg of the substance was boiled with acetic anhydride (2 ml) and freshly fused sodium acetate (25 mg) for 4 hours. The reaction product after cooling was diluted with water and then filtered. The clear solution was made ammonical by addition of dilute ammonia when the acetylated compound was precipitated. It was extracted with ether and the ethereal solution dried over anhydrous sodium sulphate. Ether was distilled off to get crude residue which upon repeated crystallization from alcohol separated as colourless needles mp. 276°C [α]D^30 = +3.6 (C 1.12 in ethanol). It did not depressed the melting point on admixture with authentic acetyl betulinic acid.

2. Benzoate

To a solution of substance (10 mg) in pyridine (2 ml), benzyol chloride (.2 ml) was added and allowed to reflux for 4 hours. The reaction product was cooled and water was added in order to decompose excess of benzyl chloride. It was then extracted with ether and the ethanol extract washed with sodium bicarbonate, dried over anhydrous sodium sulphate. Ether was then obtained was purified by crystallization from methanol needles mp. 292-5°C.
3. Methyl ester prepared with excess of ethereal solution of diazomethane it yields a crystalline needles mp. 224–25°C (α)_D = +6° (in pyridine). Found C-78.9%, H-10.6%, calculated for C_{31}H_{50}O_3 C-79.1%, H-10.6%.

4. Reduction with lithium aluminium hydride it offered betulin identical with authentic material.

**CHARACTERISATION OF COMPOUND E : (Stigmesterol)**

It is crystallized from alcohol crystalline, colourless needles obtained mp. 170°C (α)_D = 47° (chloroform). C_{29}H_{48}O Found C-84.18%, H-11.84% requires C-84.46%, H-11.65%.

**COLOUR REACTIONS**

1. When its chloroform solution was treated with 2-3 drop of concentrated sulphuric acid it produced a red colour in chloroform (Solkowski reaction).

2. A deep red colour developed when a solution of compound in chloroform was treated with concentrated sulphuric acid and few drops of acetic anhydride (Libermann-Burchard reaction).

3. When solution of compound in chloroform was mixed with acetyl chloride in excess and the little zinc chloride and then boiled - a red colour with greenish yield fluorescence developed (Tschugajev reaction).

4. The compound gave positive colour on treatment with
30% solution of the chloro-sulphonic acid in glacial acetic acid.

5. A precipitate obtained when ethanolic solution of the compound was treated with ethanolic solution of digitonin.

The above colour reaction are specific for steroids. Moreover negative Briskorne test and formation of digitonide ruled out the possibility of its being triterpenoid derivative.

**PREPARATION OF DERIVATIVE**

1. **Acetate**

   The compound (.05 gm) with anhydrous sodium acetate (.5 gm) and acetic anhydride (5 ml) was refluxed for 3 hours on a sand bath at 140°C. The contents of the flask after cooling were poured into ice cold water with constant stirring. It was kept overnight. The product obtained after filtration was washed well with distilled water and dried. The crude acetate crystallized from mixture of chloroform:methanol as colourless prism mp. 141°C. Mixed mp. of acetate derivative with that obtained from a authentic specimen of stigmesterol showed no depression.

2. **Benzoate**

   To a solution of substance (.05 gm) in pyridine
(.02 gm) benzyol chloride (.5 ml) was added and allowed to reflux for 4 hours. The reaction product was cooled and water was added in order to decompose excess of benzyol chloride. It was then extracted with ether and the ethereal extract washed with sodium carbonate, dried over anhydrous sodium sulphate, ether was then distilled off. The crude benzoate, thus obtained was purified by crystallization from methanol, needles mp. 160°C.

3. Digitonide

The compound (.05 gm) in hot absolute alcohol (5 ml) was mixed with saturated solution of digitonin in absolute alcohol (5 ml) and the mixture was heated on a water bath for 20 minutes. After cooling a precipitate separated out which was filtered and dried mp. 100°C which was not depressed by admixture with an authentic sample of digitonide derivative of stigmasterol.

CHARACTERISATION OF COMPOUND F (β-sitosterol)

The ether filtrate from the above was evaporated. The benzene:chloroform elutes gave crystalline colourless needles from alcohol mp. 137°C (α)D 22 - 37° (chloroform), C29H50O; Found C-84.31%, H-11.99%, calculated for C-84.06%, H-12.1%.

It is soluble in benzene, ether and chloroform.
but sparingly soluble in methanol.

To a solution of the substance in carbon tetrachloride a few drops of bromine solution in carbon tetrachloride, were added. The bromine colour was decolourised.

To the substance (5 mg) dissolved in chloroform one drop of tetra nitromethane (in chloroform) was added when a yellow colouration developed.

**COLOUR TESTS**

It responded to Liebermann Burchard\textsuperscript{7}, Solkowski\textsuperscript{6} and Rosenheim colour tests which are specific for the steroidal structure.

**PREPARATION OF DERIVATIVE**

1. **Acetate**

200 mg of the substance was boiled with acetic anhydride (4 ml) and freshly fused sodium acetate (500 mg) for four hours. The reaction product after cooling was diluted with water and then filtered. The clear solution was made ammonical by the addition of dilute ammonia, when the acetylated compound precipitated. It was extracted with ether and the ethereal solution dried over anhydrous sodium sulphate. Ether was distilled off to get crude residue which upon colourless needles mp. 127°C. Mixed mp. of acetate derivative with that obtained from an authentic specimen of β-sitosterol showed no
depression.

2. **Benzoate**

To a solution of substance (250 mg) in pyridine (5 ml) benzyol chloride (.5 ml) was added and allowed to reflux for four hours. The reaction product was cooled and water was added in order to decompose excess of benzyol chloride. It was than extracted with ether and the ethereal extract washed with sodium bicarbonate, dried over anhydrous sodium sulphate. Ether was then distilled off the crude benzoate thus obtained was purified by crystallization from methanol needles mp. 14°C.

3. **Digitonide**

100 mg of the substance was dissolved in hot alcohol and equal quantity of digitonin (in alcohol) was added. The mixture was warmed for about half an hour on a water bath. On cooling a flocculent precipitate was obtained. It was filtered, washed well with water and crystallized from hot ethanol, mp. 227°C which was not depressed by admixture with an authentic sample of digitonide derivative of β-sitosterol.

4. 50 mg of the substance in acetone (10 ml) was refluxed with aluminium tertiary butoxide (1 gm) in acetone for 24 hours. The solution was washed with dilute sulphuric acid then with water and dride over anhydrous sodium sulphate.
The solvent was removed and the product was chromatographed over alumina in light petroleum and eluted with benzene and ether. On evaporation of the solvent a ketone (150 mg) mp. 87°C was obtained (recorded mp. 98°C of β-sitost-4-enone 88°C).

5. 2-4-dinitrophenyl hydrazone of the above ketone

A solution of 100 mg of the ketone in 20 ml of absolute alcohol was treated with freshly prepared solution of 2-4-dinitrophenyl hydrazine and refluxed on water bath for 2 hours. It was then allowed to cool in a refrigerator overnight when a orange crystalline precipitate appeared. On recrystallization from alcohol it gave red prism mp. 250°C.

CHARACTERISATION OF COMPOUND G : (Gallotannin)

It is almost creemish, microcrystalline hygroscopic chromatographically single compound. $[\alpha]_D^{20} + 9.8$ (acetone), mp. 232-34°C which on thin layer chromatography gave Rf 0.32 (solvent-benzene:methanol (1:1), spray iodine). On paper chromatogram Rf 0.42 (solvent-butyl alcohol:acetic acid:water (4:1:5) v/v upper layer and Rf 0.82 (solvent-butyl alcohol:acetic acid:water (4:1:5) v/v lower layer. Molecular $C_{48}H_{36}O_{33} \cdot 4H_2O$.

COLOUR TESTS

1. It gave blue black colour with alcoholic ferric
chloride\(^{24}\).

2. A drop of the solution of the compound was taken on a filter paper and was sprayed with aniline hydrogen pthalate reagent, the filter paper was heated in an oven at 105\(^{\circ}\)C for about 10 minute, purple colour was obtained\(^{25,12}\).

3. To ethyl alcohol solution of compound, two drops of alkaline solution of \(\beta\)-napthol were added and concentrated sulphuric acid was added from the side of the test tube, a violet ring is formed at the junction of the two layer.

**SPECTRAL DATA**

<table>
<thead>
<tr>
<th>IR (\text{cm}^{-1})</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3450 (OH), 3000, 1720, 1600, 1450, 1370, 1050, 960, 800.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NMR (60 MHz, CDCl(_3))</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.05 (singlet 3H-(\text{C-CH}_3)), 3.95 (bs 5,4H-(\text{OCH}_3)) and six glucose protons at C-2 (1H), C-3 (1H), C-4 (1H), C-5 (1H) and C-6 (2H), 5.0 (bs 1H at C-1 of glucose), 7.3 (singlet 12H, Ar-H).</td>
<td></td>
</tr>
</tbody>
</table>

**ACETYL DERIVATIVE OF GALLOTANNIN**

The tannin (.035 gm) was dissolved in acetic anhydride (5 ml) and pyridine (1.5 ml) and the solution kept
for 48 hours at room temperature (21-25°C). It was poured over crushed ice and left overnight. The light brown solid obtained was filtered and solution was concentrated and solid portion was dissolved in it and excess of petroleum ether (40-60°) was added. The acetate was obtained as colourless solid by repeated crystallization from ethyl acetate-light petroleum mixture mp. 262-64°C (α)D 25 + 56.7 in ethyl acetate.

**ELEMENTAL ANALYSIS AND ACETYL GROUP ESTIMATION**\(^{26, 27}\)

<table>
<thead>
<tr>
<th>Found</th>
<th>Requires for C(<em>{48}H</em>{26}O_{38}) (-\text{COCH}_3)_10</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-55.00%</td>
<td>C-54.50%</td>
</tr>
<tr>
<td>H-4.00%</td>
<td>H-3.80%</td>
</tr>
<tr>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>-C-\text{CH}_3-40.34%</td>
<td>-C-\text{CH}_3-40.45%</td>
</tr>
</tbody>
</table>

**ACID HYDROLYSIS**

The tannin (.025 gm) was refluxed with alcoholic sulphuric acid (7% 25 ml) for two hours. The hydrolysate was put for continuous extraction with ether for 15 minute. The acidic solution left was neutralised with barium carbonate and tested for the presence of sugar.

**GALIC ACID**

The ether extract on paper chromatogram gave a single spot Rf 0.74 (solvent-butyl alcohol: acetic acid: water
(4:1:5) spray alcoholic ferric chloride).

**SUGAR**

The acidic solution left after extraction with ether was neutralised with barium carbonate and concentrated under reduced pressure in a rota vapour. The concentrated syrup gave positive Molisch test, reduce fehling solution and on paper chromatogram showed a single purple ring with Rf 0.58 (solvent - butyl alcohol:acetic acid:water (4:1:5) v/v, spray aniline hydrogen pthalate). Authentic sample of glucose under similar condition gave same Rf 0.58 (solvent - aniline hydrogen pthalate).

**PHENYL OSAZONE**

To the concentrated solution excess of phenyl hydrazone chloride, sodium acetate solution and saturated sodium bisulphite solution was added. On heating the mixture for about eight minute on a boiling water bath osazone appeared as deep yellow long needles mp. 203°C. The phenyl osazone compared from authentic sample of glucose had the same crystalline form and mp. 205°C.

**ALKALINE HYDROLYSIS**

The tannin (.10 gm) dissolved in alcoholic sodium hydroxide solution (5%, 5 ml) was kept at room temperature
for 24 hours but of contact with air, it was strongly acidified with hydrochloric acid and continuously extracted with ether. The ether extract of paper chromatogram Rf 0.75 (solvent-butyl alcohol:acetic acid:water (4:1:5) v/v, spray alcoholic ferric chloride) corresponding to the authentic sample of gallic acid. In the aqueous solution was found glucose Rf 0.58 on paper chromatogram (solvent-butyl alcohol:acetic acid:water (4:1:5), spray aniline hydrogen pthalate). Authentic D-glucose gave the same Rf under same condition.

**TEST FOR FREE ALDEHYDE GROUP**

Acetone, water and alcohol extracted gallotannin gave brownish purple spot on paper chromatogram when sprayed with aniline hydrogen pthalate. The ring given by methyl gallate under similar condition did not developed with reagent, hence the colour obtained with the tannin cannot be mistaken for presence of free hydroxyl is the gallic acid units. Moreover the methylation product of the tannin with diazomethane also gave the positive test. The methylated tannin also reduced, fehling solution in cold, hence it was confirmed that reducing group in glucose molecule is free.
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